

ALTERATIONS OF SEPTOHIPPOCAMPAL STRUCTURE IN
INTERLEUKIN-2 KNOCKOUT MICE

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2004

This dissertation is dedicated to my wife Laura whose love, support, and occasional nagging help to keep me focused on my goals.

ACKNOWLEDGMENTS

Traditionally, most acknowledgement sections begin by thanking one's advisor; in this case, such tradition is most warranted. As such, I thank Dr. John Petitto for being a wonderful mentor. Though not the stealthiest individual in the world with his penchant for crying out one's name (or impromptu nickname) exuberantly as soon as one enters anywhere within his field of vision, he is among the kindest, most supportive, enthusiastic, and knowledgeable mentors for which any graduate student could wish. Next, I would like to thank each member of my committee. I feel fortunate to be advised by such a great selection of knowledgeable and friendly people. Dr. Mike King's easygoing personality and extensive knowledge of all things stereological and cholinergic have proven invaluable in my studies. Dr. Mark Lewis' advice on statistics and experimental design, as well as his sense of humor, has been most appreciated. Dr. Jake Streit, in addition to his ability to make me laugh, always reminded me that there is more than one kind of cell in the brain. Dr. Mark Atkinson, always amiable and approachable, helped guide me in the "immunology" aspect of "neuroimmunology."

I also thank Dr. Huang Zhi. I cannot overstate how much I valued his advice on experiments and his daily conversations on topics ranging from basketball to politics to Hong Kong movies. I wish him the best of luck in his medical residency and his future as a psychiatrist. I would also like to thank Clive Wasserfall and Fletcher Schwartz for teaching me how to use the Luminex technology and Tim Vaught for teaching me the ins and outs of multiple microscopy techniques. I also thank the many technicians that

worked in the lab both past and present: Brent, Andrew, David, Jeannette, Dan, Jesse, and Grace. In addition to taking care of the upkeep of the lab, working with them was a pleasure. In particular, I would like to single out Andrew. He was my dearest friend here in Gainesville. I wish him the best of luck in his career as a medical doctor and hope that we will always remain friends after I leave Gainesville.

Outside of the laboratory, I thank my other friends for being my support structure. There has never been a better collection of in-the-closet geeks than Coleman, Dan, Andy, Ryan, Charles, Chris, Curtis, Nick, and Jason. They are simply the best. I would also like to thank Mozart, Michelangelo, and Sage simply because not nearly enough people thank their dogs. Obviously, they would be more likely to chew on this dissertation than read it, but I would like anyone else that does see this to know that few people are capable of matching the unconditional love that a dog has for its owner.

I also thank my family. My mother has always instilled the value of education into me. My father's love and sense of humor were crucial in the development of my personality. My sisters, Jackie and Judy, will always be among my closest friends. My Aunt Kathy and Uncle Jimmy are tremendous people who have always supported me and though they are family by marriage, our bond is stronger than blood.

Finally, I thank my lovely wife Laura. Without her, my life would be incomplete (though despite her beliefs, I could still drive effectively without her commentary from the passenger seat). She has pushed me when I needed motivation and comforted me when I need support. She is my heart and soul and I could not have achieved this dissertation without her.

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Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

ALTERATIONS OF SEPTOHIPPOCAMPAL STRUCTURE
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August 2004

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Interleukin-2 (IL-2) is a multifunctional cytokine involved in peripheral immune processes and may also be implicated in multiple brain functions. IL-2 gene knockout (IL-2 KO) mice exhibit deficits in several hippocampally-mediated behaviors (e.g., learning and memory) and have alterations in hippocampal structure.

In the first study, adult IL-2 KO and wild-type littermates were compared for differences in the cholinergic neurons in the medial septum and vertical limb of the diagonal band of Broca (MS/vDB; a structure associated with learning and memory). The IL-2 KO mice had significantly fewer cholinergic somata in the MS/vDB, but not in the striatum, thus indicating a selective effect of IL-2 on the MS/vDB. Cholinergic neurite density in the hippocampus was unaffected, but the length across the infrapyramidal (IP), but not the suprapyramidal (SP), granule cell layer (GCL) of the dentate gyrus was reduced.

The second study assayed for variations between groups in the second largest population of neurons in the MS/vDB, the GABAergic neurons. We found no differences in these neurons in IL-2 KO animals. In 21-day-old IL-2 KO mice, we detected no changes in cholinergic neuronal number in the MS/vDB. This inconsistency with adult cholinergic neurons may be due to a failure in maintenance or might be secondary to autoimmunity. Neuronal number in the IP-GCL was also decreased, consistent with the reduction in distance detected in the first study. We also discovered that IL-2 KO correlates with a hippocampal elevation in nerve growth factor (NGF), but a reduction in the brain-derived neurotrophic factor (BDNF).

Finally, in the last study, no T cells or evidence of increased activated microglia was evident in the IL-2 KO mouse hippocampus. We noted significant elevations in several cytokines (IL-12, IL-15, IP-10, MCP-1) in the hippocampus of IL-2 KO mice. The cytokine profile of the serum was different from the hippocampus, indicating that these were not global changes throughout the bodies of the animals. We also found an alteration in hippocampal neurogenesis that appeared to be attributable to differences in male mice.

The results of these studies suggest a neuroimmune interaction that may be important in septohippocampal physiology.

CHAPTER 1 BACKGROUND AND SIGNIFICANCE

Cytokine-Brain Interactions

The landmark studies of Ader and Cohen demonstrating that immune physiology could be behaviorally conditioned led to the systematic investigation of the complex interaction between the central nervous and immune systems (Ader and Cohen, 1975; Ader et al., 1982). Although the central nervous system (CNS) and peripheral immune system were once considered functionally incompatible entities separated by a nearly impermeable protective blood-brain-barrier (BBB), it is now known that there is bidirectional communication and modulation between these two systems. Cytokines have emerged as important mediators of various processes in the CNS. Their effects range from neuroinflammation in experimental autoimmune encephalomyelitis (EAE) and viral infection of the brain to neurobiological processes such as hypothalamic-pituitary axis (HPA) regulation, induction of fever, sleep, analgesia, feeding behavior, and cognition (for reviews see Ader et al., 2001; Dunn, 2002; Wilson et al., 2002).

Cytokines produced both within and outside of the CNS can exert their effect on brain cells (Dunn, 2002; Streit et al., 1998). The work of Banks and others show that the BBB acts as a selective filter for peripheral cytokines (for a review see Banks et al., 2002). Multiple studies support the ability of cytokines (e.g., IL-1 α and - β , IL-2, IL-6, IFN- α and - γ , TNF- α) to cross the BBB via different transport mechanisms (Banks et al., 1994; Banks et al., 1991; Gutierrez et al., 1993; Pan et al., 1997; Waguespack et al., 1994), and via the "leaky" circumventricular organs (CVO), four brain regions outside of

the BBB with fenestrated capillaries (Buller, 2001). Peripheral leukocytes, in particular activated T cells that enter the brain during certain conditions (e.g., EAE, facial nerve axotomy), can also release cytokines in the CNS (Hickey et al., 1991). Finally, cytokines may also interact with the brain through activation of peripheral nerves, such as IL-1 stimulation of the vagus nerve, which can lead to modulation of brain functions through its afferent connections in the CNS (Maier et al., 1998). Such a cytokine-to-nerve communication pathway may not be limited to the vagus nerve, as central hyperalgesic effects are also observed by stimulating cutaneous nerves with a subcutaneous injection of IL-1 β (Fukuoka et al., 1994), TNF- α (Sorkin et al., 1997), or antibodies against TNF- α (Lindenlaub et al., 2000). Thus, multiple pathways exist that allow cytokines to directly or indirectly influence the brain. The focus of this dissertation was on IL-2, which can be produced in the periphery and the CNS.

The Pleiotropic Cytokine: Interleukin-2

IL-2 was originally identified as a growth factor for bone marrow-derived T cells in 1976 (Morgan et al., 1976), and was renamed in 1979, when its pleiotropic effects between leukocytes (thus the term interleukin) became clear (Aarden et al., 1979). Further characterization of IL-2 revealed that it belongs to the four α -helix bundle family of cytokines; this family consists of cytokines with four α -helices connected by three loops in an up-up-down-down formation (Bazan, 1992). The receptor for IL-2 has a common gamma (γ_c) subunit shared by multiple cytokines including IL-4, IL-7, IL-9, and IL-15 (Sugamura et al., 1996); a β subunit only shared with IL-15 (Giri et al., 1995); and, in one conformation, an α subunit, which confers greater binding affinity (Leonard et al., 1984). The receptor subunits can combine in two biologically active forms: a lower

affinity heterodimer consisting of the γ_c and β subunits and a high affinity heterotrimer comprised of all three subunits (α , β , and γ_c) (Ringheim et al., 1991; Takeshita et al., 1992). The β and γ_c both possess intracellular signaling domains and in their heterodimeric form have a K_d of 10^{-9} , whereas the addition of the α subunit forms a heterotrimer with higher affinity (K_d of 10^{-11}) for IL-2 (Nakamura et al., 1994; Nelson et al., 1994).

In the peripheral immune system, where the physiological properties of IL-2 are most well-characterized, IL-2 has multiple biological functions, including natural killer (NK) cell activation, T lymphocyte activation, as well as B lymphocyte differentiation (for review see Waldmann, 2002). The creation of a transgenic knockout mouse model for this cytokine suggests that the most important function of IL-2 is the maintenance of immune self-tolerance (Schmitt et al., 1994). IL-2 knockout (KO) mice develop autoimmune symptoms commonly including inflammatory bowel disease similar to ulcerative colitis in humans and advanced hemolytic anemia, although the manifestation of the phenotype is dependent on the genetic background of the knockout mice (Horak, 1995, 1996). The autoimmunity that develops when the IL-2 gene is deleted is T cell dependent (Ma et al., 1995). More recently, it has become apparent that IL-2 plays a major role in limiting T cell responses via the development of regulatory T cells ($CD4^+CD25^+$ T reg cells) and other mechanisms that promote self tolerance and suppress T cell responses in vivo (Nelson, 2004). Though extensive research has characterized the effects of IL-2 in the peripheral immune system, increasing evidence indicates that IL-2 may potentially impact the central nervous system (CNS). The focus of this research project was to characterize these potential actions of IL-2 in the brain.

Interleukin-2 and the Brain

The effect of IL-2 on cognition and mood in humans was among the earliest findings that suggested that this cytokine might have neurobiological actions. In early clinical studies of the cognitive side effects of IL-2 therapy, 50% (i.e., 22 patients out of 44) of the subjects monitored developed cognitive changes, with 15 of them necessitating acute intervention (Denicoff et al., 1987). In addition, IL-2 therapy in patients with renal carcinoma or melanoma was found to impair spatial memory and performance in planning tasks (Capuron et al., 2001a), and induce depressive symptoms as early as two days into therapy (Capuron et al., 2000).

IL-2-brain interactions have also been investigated on an anatomical and physiological level. In landmark studies, IL-2 was found to modulate the proliferation of oligodendrocytes (Benveniste et al., 1987; Benveniste and Merrill, 1986; Saneto et al., 1986). Exogenously administered IL-2 also has multiple effects on pituitary cells including stimulation of cortisol production and adrenal corticotropin releasing hormone release (Hanisch et al., 1994), as well as increasing pituitary cell responsiveness to corticotropin-releasing hormone (Witzke et al., 2003). IL-2 has also been shown to regulate the production and secretion of peptides from hypothalamus, in addition to pituitary cells (Karanth et al., 1993; Lapchak and Araujo, 1993; Pardy et al., 1993). Subsequent research has shown that exogenously applied IL-2 can modulate other types of central nervous system cells, such as microglia (Sakai et al., 1995). Exogenously applied IL-2 can also biphasically regulate the release of some neurotransmitters such as dopamine (Alonso et al., 1993; Petitto et al., 1997), or acetylcholine (Hanisch et al., 1993; Seto et al., 1997).

IL-2 has been shown to have neurotrophic effects on cultured neurons from several regions of the rat brain including the neocortex (Shimojo et al., 1993), cortex, striatum, medial septum, and hippocampus (Awatsuji et al., 1993). Moreover, in rat hippocampal neuronal cultures, IL-2 enhances the length and branching of hippocampal neurites and the morphology of these neurons (Sarder et al., 1996; Sarder et al., 1993). Interestingly, altered levels of IL-2 expression have been detected in schizophrenia (for reviews see Hanisch and Quirion, 1995a; Muller and Ackenheil, 1998), which is a neurological disorder where altered morphology of hippocampal neurons is well documented (for a review see Thune and Pakkenberg, 2000).

IL-2-like immunoreactivity has been localized to the hippocampal formation in rat forebrain (Lapchak et al., 1991), and detected in tissue extracts from rat and human hippocampal tissue (Araujo et al., 1989). In mouse brain, IL-2 mRNA has been found in the hippocampus (Villemain et al., 1991), and transcripts for this cytokine may be expressed in rat astrocyte cultures as well (Eizenberg et al., 1995). Our lab has cloned and sequenced the full-length mouse brain cDNAs for IL-2R α as well as the IL-2/15R β and γ_c subunits, and has found that the sequences of the genes expressed by lymphocytes and in brain are identical. We have also found that these genes are enriched in the hippocampus and related limbic regions. Of particular relevance to IL-2 actions in the hippocampus, *in situ* hybridization has shown that the IL-2/15R β and γ_c genes are expressed by pyramidal and granule cell neurons (Petitto and Huang, 1994, 1995, 2001; Petitto et al., 1998).

IL-2 and the Septohippocampal System

Hippocampal circuitry is important for encoding spatial learning and memory and some evidence supports a potential role of IL-2 in the hippocampus. IL-2, for example, alters the electrophysiological characteristics of hippocampal neurons including alterations of voltage-dependent Ca^{2+} currents (Plata-Salaman and French-Mullen, 1993), depolarization and hyperpolarization of cultured hippocampal neurons (Hanisch and Quirion, 1995a), and changes in long-term potentiation (LTP) (Tancredi et al., 1990). IL-2R subunits, as previously mentioned, are enriched in the hippocampus relative to other brain regions and exogenously applied IL-2 enhances the survival and morphological development of neurons of the hippocampus.

Knockout mice deficient in IL-2 perform significantly worse than wild-type controls in one such test of spatial learning and memory, the Morris water maze; show an enhanced pre-pulse inhibition of the acoustic startle response (PPI; another hippocampally-mediated process); and also exhibit structural alterations in mossy fiber length (Petitto et al., 1999). Our initial studies suggest that this deficit in learning and memory is not likely due to a compromised immune system, as severe combined immunodeficient (SCID) mice perform significantly better than IL-2 KO mice in the Morris water maze. More recent studies from our lab suggest that the nature of the deficit in learning and memory seen in IL-2 knockout mice could be related to the immune status of the mother (normal heterozygote vs. autoimmune homozygote mother). In addition, the previously mentioned clinical studies of cancer patients under IL-2 treatment found alterations in spatial memory, lending some support to the potential role of IL-2 in learning and memory.

In the basal forebrain, the medial septum and vertical limb of the diagonal band of Broca (MS/vDB) send a large number of projections to the hippocampus, with the major neuronal phenotypes of these being cholinergic and GABAergic (Brashear et al., 1986; Kiss et al., 1990b; Kiss et al., 1990a). The septohippocampal system has been associated with learning and memory processes, with extensive data existing that link the septal cholinergic neurons that project to the hippocampus to learning and memory (Galey et al., 1994; Leanza et al., 1995), and PPI (Koch, 1996b). Moreover, variability of cholinergic fiber density in the dentate gyrus of individual mouse strains correlate with changes in spatial learning (Schwegler et al., 1996a; Schwegler et al., 1996b). Some controversy exists, however, on the relative importance of cholinergic neurons of the MS/vDB in learning and memory processes. The advent of selective toxins that target cholinergic neurons, like 192 IgG-saporin, have allowed researchers to behaviorally test animals only lacking MS/vDB cholinergic neurons, but with presumably normal distributions of GABAergic neurons. In many of these studies, animals with cholinergic septohippocampal lesions did not differ from control subjects (Baxter et al., 1996; Bizon et al., 2003; Cahill and Baxter, 2001; Chappell et al., 1998; Perry et al., 2001). Surprisingly, however, multiple other contemporary studies utilizing 192 IgG-saporin do find learning and memory deficits in the lesioned animals (Janis et al., 1998; Johnson et al., 2002; Lamprea et al., 2000; Wrenn et al., 1999). One potential explanation for this discrepancy may be that a certain threshold of cholinergic damage is necessary to elicit a deterioration in spatial learning ability (Leanza et al., 1995; Wrenn et al., 1999). Nevertheless, the negative findings are considerable and this may not explain the inconsistency well enough between groups. Another hypothesis calls into question the

efficacy of 192 IgG-saporin in completely removing septohippocampal cholinergic activity. In studies where 192 IgG-saporin appears to nearly completely eliminate cholinergic immunostaining of MS/vDB neurons, ACh release in the hippocampus of these lesioned animals still persists at ~40% of the control values (Chang and Gold, 2004; Gold, 2003). The septohippocampal cholinergic neurons are capable of compensatory collateral sprouting (Gage et al., 1983a, 1984; Gage et al., 1983b), and functional recovery of hippocampal ACh release after complete fimbria-fornix transection (Leanza et al., 1993), which may be important as a response to damage. Thus, considering the above evidence, it is clear that cholinergic input to the hippocampus plays an important role in the complex neurobiological processes of learning and memory, though it is certainly not the only system involved.

Some evidence supports a potential trophic or regulatory role for IL-2 on the cholinergic septohippocampal system. IL-2 enhances the survival of these septal and hippocampal neurons in culture (Awatsuji et al., 1993), and modulates the activity of choline acetyl transferase (ChAT) (Mennicken and Quirion, 1997). Also, IL-2 is a potent biphasic modulator of acetylcholine (ACh) release (Hanisch et al., 1993; Seto et al., 1997). At low concentrations (sub-pM), IL-2 stimulates the release of ACh, but at higher concentrations (nM), IL-2 inhibits ACh release. Since IL-2 is difficult to detect in the adult brain, endogenous brain IL-2 or IL-2 that crosses the BBB would be expected to be present at a low concentration in the normal brain.

Taking into consideration 1) the neurotrophic and neuromodulatory role of IL-2 in cultured septal and hippocampal neurons, 2) the regulatory effects of IL-2 on ACh release and ChAT activity *in vitro*, 3) the spatial learning and memory impairments of IL-2-

deficient knockout mice and cancer patients undergoing IL-2 therapy, and 4) the enhanced concentration of IL-2R subunits in the hippocampus, I hypothesized that IL-2 may play a role in the growth and differentiation of the septohippocampal cholinergic neurons.

Statement of the Problem

Normal development of septal neurons depends on trophic factors that are presumably secreted from the hippocampus. For example, the neurotrophins, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are expressed in the hippocampus and have both been shown to be important in the development, maintenance, and repair of septohippocampal neurons (Brooks et al., 1999; Conner et al., 1992; Conner and Varon, 1997; Morse et al., 1993). Perhaps, IL-2 also mediates the growth and development of septal and hippocampal neurons. This cytokine may act as a growth factor by itself, or signal the release of growth factors from neurons or glia in the hippocampal area.

In other investigations, the modulatory and growth-promoting effects of IL-2 were determined by either adding exogenous IL-2 to cultures or by injecting exogenous IL-2 into the brain (in most cases, species non-specific, e.g., human IL-2 in rats or mice). Thus, the studies utilizing exogenous IL-2 administration have several potential shortcomings: 1) the cytokine has a short half-life, and the amount of IL-2 delivered may not reflect physiologically relevant concentrations in the CNS in vivo (e.g., chronic vs. acute dosing), 2) the nature of the injections disrupts the BBB, causing potentially even more IL-2, as well as other cytokines, from the peripheral immune system to enter into the brain, 3) it is unknown during which period in neurodevelopment that IL-2 might exert these postulated effects, and 4) non-species specific IL-2 may have neurotoxic

effects, such as T and B cell invasion of the brain, angiogenesis, changes in the composition of the extracellular matrix, myelin damage, and neuronal cell loss seen in rats administered human IL-2 intracerebroventricularly via minipumps (Hanisch et al., 1996; Hanisch et al., 1997b). Thus, my approach was to use IL-2 knockout mice. These studies were the first to investigate the consequences of the absence of IL-2 on aspects of brain development and maintenance of the septohippocampal system in vivo. Our laboratory had found the aforementioned behavioral alterations in IL-2 knockout mice, and therefore another important goal of my research was to test the hypotheses regarding the neurobiological and neuroimmunological alterations that may underlie these behavioral abnormalities.

CHAPTER 2

ALTERATIONS IN SEPTOHIPPOCAMPAL CHOLINERGIC NEURONS RESULTING FROM INTERLEUKIN-2 GENE KNOCKOUT

Introduction

One of the earliest observations suggesting that cytokines could influence brain function in humans came from cancer treatment trials in which interleukin-2 (IL-2) was found to induce cognitive dysfunction and other untoward neuropsychiatric side effects in patients (Denicoff et al., 1987). Although basic research has demonstrated that IL-2 can modulate different aspects of central nervous system (CNS) function, some of IL-2's most prominent neurobiological actions occur in the hippocampal formation and related limbic regions, where receptors for this cytokine are enriched (Araujo et al., 1989; Hanisch and Quirion, 1995a; Lapchak et al., 1991; Petitto and Huang, 1994, 2001; Petitto et al., 1998).

Exogenously administered IL-2 has effects on a number of parameters of septal and hippocampal neuronal function including trophic effects on cultured fetal septal and hippocampal neurons (Awatsuji et al., 1993; Sarder et al., 1996; Sarder et al., 1993). IL-2 may also modify cellular and molecular substrates of learning and memory such as long-term potentiation (Tancredi et al., 1990), and multiple parameters of cognitive behavioral performance in animals (Bianchi and Panerai, 1993; Hanisch et al., 1997a; Lacosta et al., 1999; Nemni et al., 1992). Moreover, the neurotrophic and neuromodulatory actions of IL-2 have been implicated in abnormal hippocampal development associated with schizophrenia (Ganguli et al., 1995; Licinio et al., 1993; McAllister et al., 1995).

The potential effects of IL-2 on cholinergic neurons are particularly relevant to this study. In addition to the aforementioned trophic effects of IL-2 on cultured septal neurons, IL-2 is among the most potent modulators of acetylcholine (ACh) release from cultured septohippocampal neurons (Araujo et al., 1989; Hanisch et al., 1993; Seto et al., 1997), and can also modulate its precursor enzyme, choline acetyltransferase (ChAT) in fetal neurons (Mennicken and Quirion, 1997). Alterations in the cytoarchitecture of cholinergic septohippocampal neurons have been shown to correlate with differences in spatial learning ability in mice (Schwegler et al., 1996a; Schwegler et al., 1996b). We found that IL-2 knockout (IL-2 KO) mice exhibited impaired learning and memory performance, sensorimotor gating, and reductions in hippocampal infrapyramidal mossy neuronal fiber length (Petitto et al., 1999), a factor shown previously to correlate positively with spatial learning ability (Schopke et al., 1991; Schwegler and Crusio, 1995; Schwegler et al., 1988).

In the present study, we therefore sought to test the hypothesis that loss of IL-2 would result in abnormal neurodevelopment of septal cholinergic neurons that project to the hippocampus. Since extensive data document that these neurons play a critical role in learning and memory performance (Galey et al., 1994; Leanza et al., 1995), and given the various *in vitro* neurotrophic and neuromodulatory effects of IL-2 on developing septohippocampal cholinergic neurons, we postulated that IL-2 KO mice would have fewer cholinergic neurons in the medial septum and vertical limb of the diagonal band of Broca (MS/vDB) and a reduction in the cholinergic axonal density in the hippocampus. To accomplish this goal, IL-2 KO and wild-type littermates were compared using stereological techniques to count MS/vDB cholinergic somata stained with ChAT

immunohistochemistry, and image analysis methods to measure the density and distribution of cholinergic neurites in several regions of the hippocampus labeled for acetylcholine esterase (AChE), a reliable marker of cholinergic axons (Hedreen et al., 1985).

Materials and Methods

Animals and Tissue Preparation

Mice used in these experiments were cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Mice were bred in our colony using IL-2 heterozygote by IL-2 heterozygote crosses. The polymerase chain reaction (PCR) was used to genotype the offspring post-weaning (see below). The IL-2 KO mice, obtained originally from the NIH repository at Jackson Labs, were derived from ten generations of backcrossing onto the C57BL/6 background. Mice were housed under specific pathogen-free conditions. Animals used in these experiments were 8-12 weeks of age.

Each animal was anesthetized with sodium pentobarbital (50 mg/kg) and perfused with 0.9% saline followed by 4% paraformaldehyde in phosphate buffered saline (PBS). The brains were removed and fixed overnight in 4% paraformaldehyde followed by overnight equilibration in 30% sucrose cryoprotective solution, and then were snap frozen in isopentane (-80°C) for storage. The brains were equilibrated to -20°C prior to cryostat sectioning into 40 µm slices in the coronal plane, collected into individual wells of polystyrene 24-well plates (NUNC 1147), and stored free-floating at 4°C in PBS for histochemistry. Every third section was processed for ChAT immunohistochemistry, AChE histochemistry, or cresyl violet Nissl staining.

Genotyping Using PCR

The genotypes of all mice were determined by the PCR. PCR reactions were performed using a 25 µl total reaction volume containing 1 µM each of forward and reverse primers, 0.1 µg genomic DNA, 0.2 mM of each dNTP, 0.3 µl Taq DNA polymerase and amplified using a thermal cycler with a heated evaporation cover (Ericomp). The cycling parameters were hot start 95°C (3min), denaturing 94°C (30 sec), annealing 64°C (30 sec), extension 72°C (45 sec) with a final extension step of 4 min. Thirty cycles were used for these experiments. The 5' and 3' primers for the IL-2 KO (500 bp knockout band amplified) were 5'-TCGAATCGCCAATGACAAGACGCT-3' and 5'-GTAGGTGGAAATTCTAGCATCATCC-3'. The 5' and 3' primers for the wild type (324 bp wild type band amplified) were 5'-CTAGGCCACAGAATTGAAAGATCT-3' and 5'-GTAGGTGGAAAATTCTAGCATCATCC-3'.

ChAT Immunohistochemistry

Free-floating 40-µm sections were incubated for 20 minutes in 1% hydrogen peroxide (H₂O₂) to quench endogenous peroxidative activity. The sections were then washed twice in PBS and blocked for 1 hr in 200 µl/well 3% normal goat serum (NGS). After this incubation, the sections were incubated overnight in the primary antibody, rabbit anti-ChAT (Chemicon AB143; 1:2000 dilution in PBS with 0.3% Triton X-100 and 1% NGS, 200 µl/well). The next day, the sections were washed twice in PBS and incubated overnight in the secondary antibody, biotinylated goat anti-rabbit IgG (Sigma B-7389; 1:1000 dilution in PBS with 0.3% TX-100 and 1% NGS). The sections were then washed twice in PBS and incubated in ExtrAvidin (Sigma E-2886; 1:1000 in PBS)

for 2 hrs followed by two washes in PBS. The sections were developed in 0.5 mg/ml 3,3'-diaminobenzidine (DAB), 0.2 mg/ml urea H₂O₂ for approximately 5 min and were placed on slides, dehydrated in graded ethanol washes, cleared in two changes of xylenes, and coverslipped. Figure 2-1 shows an example of ChAT immunostained section of the MS/vDB.



Figure 2-1. ChAT immunohistochemical staining of the medial septum/vertical limb of the diagonal band of Broca. The scale bar represents 200 μ m.

AChE Histochemistry

AChE histochemistry was used as a marker of cholinergic innervation of the hippocampus (Woolf et al., 1984). Brain sections were collected in individual wells of 24-well plates containing 250 μ l/well 0.1 M pH 6.0 acetate buffer (AB). The sections were washed twice with AB, then placed in 200 μ l preincubation solution consisting of aqueous 5 mM sodium citrate, 3 mM cupric sulfate, and 0.5 mM potassium ferricyanide. The sections were incubated for 20 minutes at room temperature on a shaker at a low speed. After the preincubation period, 200 μ l of the incubation solution was added to each well consisting of the same make-up as the preincubation solution supplemented with 4.84 mM acetylthiocholine iodide and 0.4 mM ethopropazine. The multi-well plate was packed on top of crushed ice and microwaved at 200 W for 2 minutes. The solution was then removed and the sections were washed twice in 0.05 M TRIS pH 7.6 buffer followed by AB. The reaction product was intensified with 0.5 mg/ml DAB, 2.5% nickel sulfate, and 0.01 % H_2O_2 in AB for 5-7 min or until definitive staining could be detected in the hippocampal subregions. Sections were then mounted on slides, dehydrated in grade ethanol washes, cleared in xylenes, and coverslipped for imaging.

Cholinergic Stereology

Stained cholinergic neuronal somata of the MS/vDB were counted using the software MCID 5.1 and the three-dimensional counting box (optical dissector) method described by Williams and Rakic (Williams and Rakic, 1988). All stereology was performed using a CCD High Resolution Sony camera and a Zeiss Axioplan 2 microscope with a motorized x-y stage made by Imaging Research, Inc. The latter is capable of making movements as fine as 0.1 μ m. Every third section through the anterior-posterior extent of the septal region was sampled. The regions to be counted

were outlined at 10x magnification and the size of the counting boxes were generated to be approximately 5% of the most rostral, and therefore, smallest, area of the MS/vDB (defined by the section where the corpus collosum first joins in the midline). The size of the outlined count regions, but not the counting box, varied depending on where the individual section was taken from the rostral to caudal extent of the MS/vDB. The defined counting box was approximately 2-2.5% of the outlined count area of the largest single section of the MS/vDB.

To assess whether the predicted septal cholinergic alterations in IL-2 KO mice might be associated with a general effect on cholinergic neurons in the brain, striatal cholinergic somata were also counted in the right hemisphere in the sections that also contained the MS/vDB. Except for a different magnification used to outline the striatum (5X), the sampling parameters were identical to those used to generate estimates of septal cholinergic neuron number. The guard volume was set at 1 μm for the top and bottom of the section and the counting cubes were randomly distributed throughout the user-defined count area with a total sampling frequency of 25% (~8.3% of the total area since every third section was sampled). Only somata that were clearly and distinctly stained were counted. Each counting box was examined at 40x magnification (20x for striatal neurons) and the computer-assisted focus was used to scan from the top to the bottom of the counting box. Cells were counted only if they were either completely inside of the counting box, or partially inside of the box on the top, back, or left side. They were not counted if they fell outside of the box or crossed into the box anywhere on the bottom, front, or right side. The cells that were counted were labeled on the monitor by clicking the mouse pointer on each cell and the MCID software recorded the number of marks.

The MCID software interpolated the total volume of the MS/vDB based on the volume of the count areas defined by the user. Cell density (N_v) was estimated by dividing the total number of cells counted by the volume of the counting boxes, which was also tracked by the software. The total cell number was estimated by multiplying N_v by the total volume.

Quantitative Image Analysis of AChE Staining

For quantitative analysis of AChE staining, we modified previously described methods used to measure intensity of staining and comparisons of normalized length across CA1, CA3b, and the suprapyramidal (SP) and infrapyramidal (IP) blades of the dentate gyrus (DG) (King et al., 1989; Schwegler et al., 1996b). Images of the hippocampus in each tissue section sampled from a light microscope (Olympus BH-2) were relayed by digital video camera (Hitachi KP-D581) to a computer frame grabber (Flashpoint 128, Integral Technologies) and digitized to 640x480 pixel images with 256 gray levels from black to white. Imaging software (Image Pro Plus v.4.0, Media Cybernetics) was utilized to define a broad sampling traverse across various areas of the hippocampus, approximately perpendicular to the cell body layers. For CA1 and CA3b, this traverse extended from the alveus to the hippocampal fissure. For the DG, the line extended from the hilus to hippocampal fissure or to the pial surface for the SP or IP limb of the DG, respectively. AChE-containing fiber density was estimated by using the gray level of each point along the line, which was calculated by averaging the intensity value of each pixel across the width of the sampling band (e.g. a traverse with a width of 46 pixels would have an average of 46 measurements approximately parallel to each data point). Gray level intensity measurements were used to estimate the AChE reaction product density at each point along the traverse. The mean pixel intensity of a small box

positioned in the corpus collosum was used to represent the background staining intensity of tissue containing little cholinesterase activity. All of the average intensities along the line were then plotted by traverse position to illustrate the quantitative patterns of AChE distribution across each subregion. Conspicuous inflections marking the transition from alveus (Alv) to stratum oriens (SO, in CA1, CA3b), from stratum lacunosum-moleculare (Lmol) to dentate molecular layer (Mol) (hippocampal fissure (HiF); CA1, CA3, DG), and polymorph zone (PoDG) to granule cell layer (GCL) were used to align traverses across sections and animals (Figure 2-2). Also, length-normalized comparisons of the

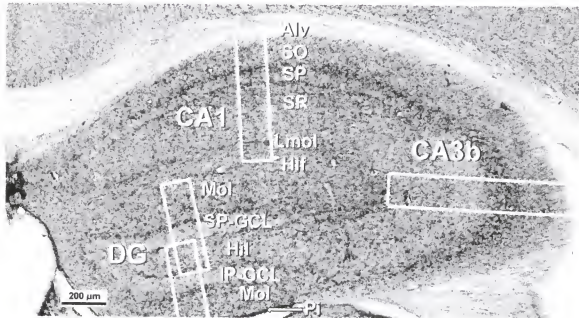


Figure 2-2. A micrograph of the sampling regions utilized for image analysis of AChE-staining. Alv=alveus, DG=dentate gyrus, GCL=granular cell layer (IP=infrapyramidal, SP=suprpyramidal), Hif=hippocampal fissure, Hil=hilus, Lmol=lacunosum moleculare, Mol=molecular layer, Pi=pial surface, SO=stratum oriens, SP=septum pellucidum, SR=stratum radiatum. The scale bar represents 200 μm.

measured regions were made by converting the sampling lengths to 100 points using the software Matlab v.5.3. Dependent variables were the absolute length of traverses, AChE intensity at anatomically identifiable inflection points (IP and SP bands, Lmol, dentate

inner Mol) and subregions (SO, stratum radiatum (SR), dentate outer Mol), and derived values for absolute and relative positions of, and distances between, these landmarks. For each animal, three hippocampal slices were measured on both the left and right hemispheres of the brain and the measured intensities or distances were averaged together for statistical analysis.

Cresyl Violet Staining

Every third section was Nissl stained to provide a qualitative view of the boundaries between various forebrain regions. The sections were placed on slides and allowed to air-dry. The slides were immersed in 60°C cresyl violet for 45 sec, washed in running distilled water to remove the excess cresyl violet, dehydrated in graded ethanol, cleared in xylenes, and coverslipped.

Results

Comparison of Cholinergic Somata in the MS/vDB

Figure 2-3 shows the total number of ChAT-positive cells stereologically counted from the MS/vDB of IL-2 KO and wild-type mice. As seen in this figure, the IL-2 KO mice had approximately 26% fewer cholinergic somata in this region than wild-type controls. An ANOVA confirmed that this group difference was statistically significant ($F(1,16)=8.6$, $p=.01$). By contrast, the number of ChAT-positive somata in the striatum of IL-2 KO and wild-type mice were not different. There were no significant gender differences in either brain region.

Density of AChE-positive Fibers in Regions of the Hippocampus

The average AChE-staining intensity curves were generated by defining a region across CA1, CA3b, and the SP and IP blades of the DG. Figure 2-4 shows the intensity curves that were generated from imaging the AChE-histochemically stained sections for

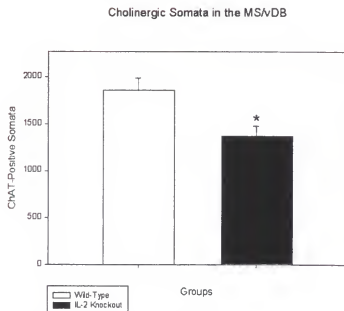


Fig. 2-3. ChAT-positive somata are significantly reduced in the MS/vDB of IL-2 knockout mice. Each bar represents the mean $\pm$ SEM of 9 animals per group. * $p=0.01$.

CA1 (Figure 2-4a), CA3 (Figure 2-4b), the SP-GCL (Figure 2-4c), and IP-GCL layer of the DG (Figure 2-4d). Repeated measures ANOVA was performed on regions of the average normalized curves selected by areas that appeared to deviate between the groups. None of these areas, however, were found to differ between IL-2 KO and wild-type mice.

Morphology of the Granular Cell Layer of the Lower Limb of the DG

There were no differences in the groups for the Y-axis (intensity) data. The variations in the patterns of the DG curves X-axis (distance) were also compared. Distances were compared by defining the point of lowest intensity in the regions that the curves indicated as each transition between the regions of interest. Distances are reported as a percentage of the total distance across each curve. The IP blade of the DG was broken into three regions: the Hil, the IP-GCL, and the Mol (Figure 2-4d). As depicted in

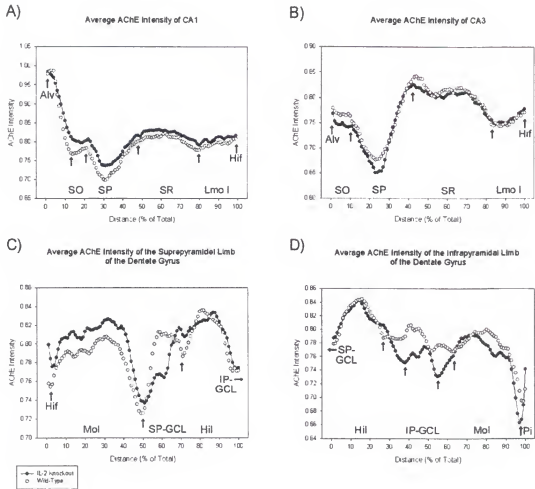


Fig.2-4. IL-2 knockout mice do not differ in measures of average intensity of AChE-staining across CA1, CA3b, and the suprapyramidal and infrapyramidal layers of the DG. Each curve represents the mean of 9 wild-type (open circles) or 9 IL-2 knockout mice (closed circles). Alv=alveus, GCL=granular cell layer (IP=infrapyramidal, SP=suprapyramidal), Hif=hippocampal fissure, Hil=hilus, Lmol=lacunosum moleculare, Mol=molecular layer, Pi=pial surface, SO=stratum oriens, SP=septum pellucidum, SR=stratum radiatum. A) The CA1 curve shows the average intensity from the Alv to the Hif. The arrows delineate the transitions between the hippocampal substructures including the SO, SP, SR, and Lmol. B) The pattern of the peaks and valleys of the CA3 region curve is similar to that of the CA1. C) The intensity curve of SP-GCL begins at the Hif and terminates at the dorsal border of the IP-GCL. Arrows delimit the borders of the Mol, SP-GCL, and Hil. D) The curve representing the IP-GCL begins at the ventral border of the SP-GCL and continues to the pial surface. The second and third solid arrow define the borders in wild-type mice between the Hil and IP-GCL and the IP-GCL and Mol, respectively. The broken arrows define these same borders in IL-2 knockout mice.

Figure 2-5, the distance across the IP-GCL was significantly reduced in the IL-2 KO mice compared to wild-type mice ($F(1,16)=9.2$, $p=0.008$). The distances across the Hil and Mol, however, were not significantly different. The SP blade of the DG was separated into three regions: the Mol, the SP-GCL, and the Hil (Figure 2-4c). There were no significant differences in length between groups across any of the internal blade regions.

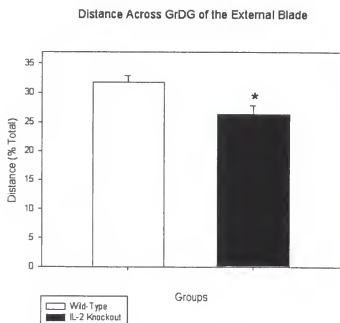


Fig. 2-5. Distance across the GrDG of the external blade was significantly decreased in IL-2 knockout mice compared to wild-type mice. Each bar represents the mean $\pm$ SEM of nine animals per group. * $p=0.008$.

Discussion

These data are the first to demonstrate that loss of endogenous IL-2 results in reduction in the number of MS/vDB cholinergic neurons and structural alterations in the morphology of the dentate gyrus. Given the role of septohippocampal cholinergic neurons in learning and memory (Galey et al., 1994; Leanza et al., 1995; Schwegler et al., 1996a; Schwegler et al., 1996b), sensorimotor gating (Caine et al., 1992; Curzon et al.,

1994; Koch, 1996a), and the aforementioned influence of IL-2 on parameters of cholinergic function, the present findings are consistent with alterations in these behavioral measures that we have reported previously in IL-2 KO mice (Petitto et al., 1999).

In this study, the number of cholinergic cell bodies in the MS/vDB of wild-type mice were comparable to those reported previously for C57BL/6 mice (Schwegler et al., 1996b). Cholinergic somata were reduced by 26% in IL-2 KO mice as compared to wild-type mice. This was not a general effect on cholinergic neurons in the brain, however, as striatal ChAT-positive neurons were not significantly affected. This finding is also consistent with previous research showing that exogenous IL-2 has potent effects on ACh release from septohippocampal neurons, whereas cholinergic interneurons in the striatum do not respond to IL-2 (Hanisch et al., 1993). One potential caveat of using ChAT as a marker of cholinergic neurons is that differences in cell counts may be due to a decrease in ChAT labeling intensity rather than a reduction in cholinergic neurons (Ward and Hagg, 2000). This would appear unlikely, however, as we did not note any appreciable differences in the staining intensity between groups.

Alterations in AChE-staining intensity did not differ as we hypothesized. It is not likely that the unexpected lack of difference in AChE-staining intensity would be attributable to the length normalization technique used, as the patterns of the average intensity curves seen in Figure 2-4 were remarkably similar between the groups. This unexpected result may, however, be due to compensatory sprouting of the surviving MS/vDB neurons during development. Indeed, numerous other studies have found that septohippocampal neurons can undergo compensatory sprouting in response to injury

(Cassel et al., 1997; Gage and Bjorklund, 1987; Gage et al., 1984; Gage et al., 1983b), and in animal disease models such as Alzheimer's transgenic mice (Bronfman et al., 2000). Future studies are needed to address this issue more directly.

Another significant finding of the current study was that IL-2 KO mice exhibited structural alterations in the distance across the IP-GCL. The neurons of the GCL have been associated with learning and memory (Collier and Routtenberg, 1984; Conrad and Roy, 1993; McLamb et al., 1988; Nanry et al., 1989; Walsh et al., 1986), and are also a target for septohippocampal cholinergic axon termination (Makuch et al., 2001). In situ hybridization studies have found the GCL to be enriched in IL-2 receptors (Petitto and Huang, 2001; Petitto et al., 1998), supporting a possible role for IL-2 in the observed structural alterations. Also, GCL development progresses from the SP layer to the IP layer (Bayer, 1980). The differences seen in the IL-2 KO mice in this study may indicate a failure of these late stage granule cells to fully develop or survive. Whether these structural changes are due to a reduced number of GCL cells or a decrease in the cell body size of these neurons requires further investigation.

The most likely mechanism whereby loss of IL-2 results in these changes in the septohippocampal cholinergic system would appear to be due to the absence of its neurotrophic actions during development. As noted earlier, IL-2 enhances neurite extension and survival of cultured fetal septal and hippocampal neurons (Awatsuji et al., 1993; Hanisch and Quirion, 1995a; Sarder et al., 1996; Sarder et al., 1993), and thus, the absence of these intrinsic effects of IL-2 could account for the observed neuroanatomical alterations. Another mechanism that may account for these findings is the possibility that the loss of endogenous IL-2 may result in lower levels of tonic ACh release during

critical periods of neurodevelopment. Release of ACh by developing neurons has been shown to be important for growth cone guidance (Zheng et al., 1994), neuronal growth and differentiation, synaptic plasticity (Lauder and Schambra, 1999), and survival of newly developed neurons (Knipper and Rylett, 1997). In fact, some evidence indicates that ACh released from developing neurons may engage in a positive feedback mechanism with nerve growth factor (NGF) (Knipper et al., 1994), a member of the neurotrophin family that is essential for the normal development of septal cholinergic neurons (Arimatsu and Miyamoto, 1991; Hartikka and Hefti, 1988; Mobley et al., 1986; Ruberti et al., 2000). In a series of studies, Quirion's laboratory has demonstrated that IL-2 is among the most potent modulators of ACh release from mature brain slices and fetal neurons *in vitro*, and can upregulate ChAT in fetal septal neurons in culture (Hanisch et al., 1993; Mennicken and Quirion, 1997; Seto et al., 1997). It is therefore possible that the loss of such potent actions of IL-2 during development could account, in part, for the cytoarchitectural alterations found in this study. Indeed, both IL-2's neurotrophic effects and action on cholinergic release may well be operative and interactive with one another. Nevertheless, the IL-2 KO mice do not exhibit complete loss of septal cholinergic neurons suggesting that the effects of IL-2 on MS/vDB neurons are likely secondary to other trophic factors like NGF.

These experiments do not enable us to differentiate between the contributions of the loss of central versus peripheral IL-2, and thus, it remains to be determined whether these septohippocampal cholinergic abnormalities are due primarily to the absence of central, peripheral, or a combination of both sources of IL-2. There is some evidence that endogenous IL-2 may be produced in neuronal areas of the mammalian hippocampal

formation, where its release may regulate the development and function of septal cholinergic neurons projecting to the hippocampus. IL-2-like immunoreactivity has been localized to the hippocampal formation in rat forebrain (Lapchak et al., 1991), and detected in tissue extracts from rat and human hippocampal tissue (Araujo et al., 1989). In mouse brain, IL-2 mRNA has been found in the hippocampus (Villemain et al., 1991), and transcripts for this cytokine may be expressed in rat astrocyte cultures as well (Eizenberg et al., 1995).

In the periphery, absence of endogenous IL-2 leads to an immunodysregulation that produces loss of self-tolerance and IL-2 KO mice eventually develop generalized systemic autoimmune disease (although C57BL/6-IL-2 KO mice develop clinical signs of systemic autoimmunity at a substantially slower rate than other strains such as Balb/c or C3H) (Petitto et al., 2000). Therefore, it is reasonable to speculate that the neuroanatomical alterations found in the IL-2 KO mice result from peripheral autoimmune processes. Autoimmunity could impact on brain development or induce neurodegeneration. The former seems unlikely, however, since IL-2 KO mice do not express the first signs autoimmunity (e.g., splenomegaly) until at least three to four weeks after birth (Horak, 1995); by this time, septohippocampal development should already be complete (Bender et al., 1996; Chandler and Crutcher, 1983; Super and Soriano, 1994; Yoshida and Oka, 1995). Furthermore, the likelihood that these neuroanatomical alterations may be due to autoimmune-induced degeneration of existing neurons also seems unlikely, since lymphocytes cannot be detected in the brain of adult IL-2 KO mice (Petitto et al., 1999). Nonetheless, since autoimmunity has been associated with cognitive changes in both animals and humans (Lal and Forster, 1988; Sakic et al., 1997;

Sakic et al., 1993), more subtle autoimmune processes may be at play in the IL-2 KO mice (e.g., autoantibodies). It would be of interest to explore the observed cholinergic changes in old versus neonatal mice to determine if the abnormalities increase with age due to neurodegeneration, or are primarily the result of abnormal development. Such knowledge will then enable us to develop a more specific model to test relevant hypotheses involving IL-2 at specific anatomical sites in the septohippocampal cholinergic system.

In summary, these data demonstrate that loss of endogenous IL-2 results in reduction in the number of cholinergic neurons in the MS/vDB and alterations in the structural morphology of dentate projection fields. These findings extend our previous experiments showing that spatial learning and hippocampal mossy fiber length are abnormal in IL-2 KO mice (Petitto et al., 1999). Further research is needed to determine whether these outcomes in IL-2 KO mice may be due to the absence of central or peripheral IL-2 during neurodevelopment (or some combination of both sources), neurodegeneration secondary to peripheral autoimmunity, or other factors associated with the absence of IL-2.

CHAPTER 3

ALTERED HIPPOCAMPAL STRUCTURE AND NEUROTROPHIN LEVELS IN INTERLEUKIN-2 KNOCKOUT MICE

Introduction

Interleukin-2 (IL-2) has been implicated in the pathogenesis of multiple sclerosis and several major neuropsychiatric disorders such as Alzheimer's disease, schizophrenia, and Parkinson's disease (Hanisch and Quirion, 1995b). Furthermore, in case studies of humans receiving IL-2 treatment for cancer therapy, prolonged exposure to IL-2 was found to induce cognitive dysfunction and other untoward neuropsychiatric side effects (Denicoff et al., 1987). Although IL-2 has been shown to be capable of modulating different aspects of central nervous system (CNS) function, many of its known effects in the limbic system occur in the hippocampal formation, where receptors for this cytokine are enriched (Araujo et al., 1989; Hanisch and Quirion, 1995a; Lapchak et al., 1991; Petitto and Huang, 1994, 2001; Petitto et al., 1998). IL-2 may, for example, modify cellular and molecular substrates of learning and memory such as long-term potentiation (Tancredi et al., 1990), and can affect multiple parameters of cognitive behavioral performance in animals (Bianchi and Panerai, 1993; Hanisch et al., 1997a; Lacosta et al., 1999; Nemni et al., 1992). IL-2 can provide trophic support to primary cultured neurons from multiple region of the rat brain, including the hippocampus and medial septum (Awatsuji et al., 1993; Sarder et al., 1993), and positively affects the morphology of neurite branching from rat hippocampal cultures (Sarder et al., 1996; Sarder et al., 1993). Furthermore, IL-2 has been shown to be one of the most potent modulators of

acetylcholine (ACh) release from rat hippocampal slices (Hanisch et al., 1993; Seto et al., 1997), and can also increase the activity of its precursor enzyme, choline acetyltransferase (ChAT) (Mennicken and Quirion, 1997).

Previously, we found that IL-2 knockout mice (IL-2 KO) exhibited impaired learning and memory performance, sensorimotor gating, and reductions in hippocampal infrapyramidal mossy neuronal fiber length (Petitto et al., 1999), a factor which correlates positively with spatial learning ability (Schopke et al., 1991; Schwegler and Crusio, 1995; Schwegler et al., 1988). We also found in studies of IL-2 KO mice *in vivo*, there was a marked reduction in cholinergic somata in medial septal/vertical limb of the diagonal band of Broca (MS/vDB) region, as well as decrease in the distance across the infrapyramidal granule cell layer (IP-GCL) of the dentate gyrus (DG) (Beck et al., 2002). Variation in the cytoarchitecture of cholinergic septohippocampal neurons correlate with differences in spatial learning ability in mice (Schwegler et al., 1996a; Schwegler et al., 1996b).

Research has shown that the neurotrophins, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) expressed in the hippocampus, can be important in the development, maintenance, and repair of septohippocampal neurons *in vitro* (Arimatsu and Miyamoto, 1991; Conner and Varon, 1997; Gahwiler et al., 1987; Hartikka and Hefti, 1988; Morse et al., 1993). Similar trophic effects have been noted in studies utilizing infusion of exogenous neurotrophins *in vivo* (Hagg et al., 1990; Morse et al., 1993), and in studies of transgenic and knockout mice (Ruberti et al., 2000; Ward and Hagg, 2000). In the peripheral immune system of multiple animal species, both NGF and BDNF are expressed by T lymphocytes (Braun et al., 1999; Kerschensteiner et al., 1999;

Mizuma et al., 1999; Moalem et al., 2000). Though IL-2 regulates several aspects of T cell function, the production or release of NGF and BDNF from T lymphocytes by IL-2 has not been tested directly (Carter et al., 1998; He and Malek, 1998), and conversely, it is not known whether NGF and BDNF can modulate IL-2 in the brain.

In the present study, we sought to expand our previous findings that loss of endogenous IL-2 in knockout mice led to reductions in cholinergic neurons of the MS/vDB and a decrease in the distance across the IP-GCL of the DG. First, we compared parvalbumin (Parv)-labeled somata in 8-12 week old IL-2 KO and wild-type littermates to test the hypothesis that the loss of IL-2 is selective for cholinergic, but not GABAergic cell bodies loss in the MS/vDB (e.g., not a general effect occurring on all neurons in this region of the brain). Second, we compared cholinergic MS/vDB somata between younger wild-type and IL-2 KO mice at postnatal day 21 (P21), an age where septohippocampal development in mice is nearly complete (Armstrong et al., 1987; Gould et al., 1991; Makuch et al., 2001). This age also precedes the development of autoimmune disease in IL-2 KO mice of the C57BL/6 background, e.g., absence of splenomegaly, lymphadenopathy, and inflammatory bowel disease. Third, we sought to expand on the previous finding that there was a reduction in distance across IP-GCL by performing stereological cell counts of Nissl-stained dentate gyri of IL-2 KO and wild-type littermates to determine if the reduction in distance could be contributed to a reduction in granule cell number. Finally, we tested the hypothesis that loss of IL-2 may impact the expression and release of the neurotrophins, NGF and BDNF, which may contribute to the MS/vDB cholinergic and GCL deficits that we observed previously.

Methods

Animals and Genotyping

Mice used in these experiments were cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Mice were bred in our colony using IL-2 heterozygote by heterozygote crosses. The IL-2 KO mice, obtained originally from the NIH repository at Jackson Laboratories, were derived from ten generations of backcrossing onto the C57BL/6 background. Mice were housed under specific pathogen-free conditions. Animals used in these experiments were either 21-days-old (for ChAT immunohistochemistry) or 8-12 weeks of age. All experiments were performed with independent groups of animals. The specific animal numbers utilized are reported at the beginning of each method descriptions below.

The genotypes of all mice were determined by the polymerase chain reaction (PCR). PCR reactions were performed using 25 μ l total reaction volume containing 1 μ M each of forward and reverse primers, 0.1 μ g genomic DNA, 0.2 mM of each dNTP, 0.3 μ l Taq DNA polymerase, and amplified using a thermal cycler with a heated evaporation cover (Ericomp). The cycling parameters were hot start 95°C (3min), denaturing 94°C (30 sec), annealing 64°C (30 sec), extension 72°C (45 sec) with a final extension step of 4 min. Thirty cycles were used for these experiments. The 5' and 3' primers for the IL-2 KO (500 bp knockout band amplified) were 5'-TCGAATCGCCAATGACAAGACGCT-3' and 5'-GTAGGTGGAAATTCTAGCATCATCC-3'. The 5' and 3' primers for the IL-2 wild type (324 bp wild type band amplified) were 5'-

CTAGGCCACAGAATTGAAAGATCT-3' and 5'-
GTAGGTGGAAAATTCTAGCATCATCC-3'.

Immunohistochemistry

For Parv stereological cell counts, six animals per group were used and for 21-day-old ChAT stereological cell counts, seven animals per group were used. Each animal was anesthetized with an injection cocktail of 3:3:1 ketamine (100 mg/ml): xylazine (20 mg/ml): acepromazine (10 mg/ml) at a dose of 0.015 ml injection cocktail/g body weight and perfused with 0.9% saline followed by 4% paraformaldehyde in phosphate buffered saline (PBS). The brains and spleens were removed and fixed overnight in 4% paraformaldehyde, followed by overnight equilibration in 30% sucrose cryoprotective solution, and then, were snap frozen in isopentane (-80°C) for storage. The spleens were weighed to assay for relative splenomegaly of IL-2 KO vs. wild-type mice. The brains were equilibrated to -20°C prior to cryostat sectioning into 50 µm slices in the coronal plane, collected into individual wells of polystyrene 24-well plates (NUNC 1147), and stored free-floating at 4°C in PBS for histochemistry. Every third section was processed for Parv or ChAT immunohistochemistry, or cresyl violet Nissl staining.

Free-floating 50-µm sections were labeled for Parv and ChAT-immunohistochemistry as described previously (Beck et al., 2002). Briefly, they were incubated for 20 minutes in 1% hydrogen peroxide (H₂O₂) to quench endogenous peroxidative activity. The sections were then washed and blocked for 1 hr in 200 µl/well 3% normal goat serum (NGS). After this incubation, the sections were incubated overnight in the primary antibody, rabbit anti-ChAT (Chemicon; 1:2000 in PBS with 0.3% Triton X-100 and 1% NGS, 200 µl/well) or rabbit anti-Parv (Chemicon; 1:1000 in PBS with 0.3% Triton X-100 and 1% NGS, 200 µl/well). The next day, the sections were

washed and incubated overnight in the secondary antibody, biotinylated goat anti-rabbit IgG (Sigma B-7389; 1:1000 dilution in PBS with 0.3% TX-100 and 1% NGS). The sections were then washed and incubated in ExtrAvidin (Sigma E-2886; 1:1000 in PBS) for 2 hrs. The sections were developed in 0.5 mg/ml 3,3'-diaminobenzidine (DAB), 0.2 mg/ml urea H₂O₂ for approximately 5 min and were placed on slides, dehydrated in graded ethanol washes, cleared in two changes of xylenes, and coverslipped.

Cresyl Violet Staining

For stereological cell count of Nissl-stained granule cells, seven animals per group were used. The tissue for this assessment was selected, because it originated from animals utilized in a previous study of cholinergic differences in IL-2 KO mice (Beck et al., 2002). Every third section was Nissl stained to provide a qualitative view of the boundaries between various forebrain regions, as well as labeling of hippocampal granule cells layers (GCL) for stereological counting. The sections were placed on slides and allowed to air-dry. The slides were immersed in 25°C cresyl violet for 10 min, washed vigorously in rapid exchanges of distilled water to remove the excess cresyl violet, dehydrated in graded ethanol, cleared in xylenes, and coverslipped.

Stereology

Stained neuronal somata of the MS/vDB or IP and SP-GCL were counted using the software MCID 5.1 as previously described (Beck et al., 2002). All stereology was performed using a CCD High Resolution Sony camera and a Zeiss Axioplan 2 microscope with a motorized x-y stage made by Imaging Research, Inc. Every third section through the anterior-posterior extent of the MS/vDB or IP and SP-GCL regions were sampled. The regions to be counted were outlined at 10x (MS/vDB) or 20x (IP and SP-GCL) magnification and the size of the counting boxes were generated to be

approximately 2-2.5% of the outlined count area of the largest single section of the areas of interest.

The rostral border of the GCL count area was defined as the first section where the dentate granule cell layer clearly separated from the pyramidal layer of CA1. The caudal border was defined as the first section where the habenular commissure was visible in the third ventricle. Only cells that could clearly be determined to be part of either the IP or SP-GCL were counted; any cells in the area where the IP and SP-GCL connected were left uncoun ted. Furthermore, only Nissl-stained cells with clearly visible nucleoli were counted. For the MS/vDB, the rostral border was determined as the first section where the corpus collosum connected in the midline of the section and the caudal border was the first section where the anterior commissure joined in the midline.

The guard volume was set at 2 μm for the top and bottom of the section and the counting cubes were randomly distributed with a total sampling frequency of the outlined count area of 25% for MS/vDB and 33% for IP and SP-GCL (~8.3% and 11% of the total area respectively, since every third section was sampled). The outlined counting area was defined by the user and only somata that were clearly and distinctly stained were counted. Each counting box was examined at 40x magnification and the computer-assisted focus was used to scan from the top to the bottom of the counting box. Cells were counted only if they were either completely inside of the counting box, or partially inside of the box on the top, back, or left side. They were not counted if they fell outside of the box or crossed into the box anywhere on the bottom, front, or right side. The cells that were counted were labeled on the monitor by clicking the mouse pointer on each cell and the MCID software recorded the number of marks.

The MCID software interpolated the total volume of the MS/vDB based on the volume of the count areas defined by the user. Cell density (N_v) was estimated by dividing the total number of cells counted by the volume of the counting boxes, which was also tracked by the software. The total cell number was estimated by multiplying N_v by the total volume.

Enzyme-linked Immunosorbent Assay (ELISA) Characterization of NGF and BDNF

For measurement of BDNF in hippocampus and MS/vDB, nine IL-2 KO and seven wild-type mice were used. For measurement of NGF protein levels, seven animals per group were used. Initial test runs revealed that some, but not all, of the NGF protein levels fell below the sensitivity of detection for the kit; therefore, the homogenized NGF samples were spiked with 25 pg/ml of the known NGF standard included with the kit to bring any low levels of expression above the kit's 15.6 pg/ml lower detection limit. One column of the ELISA plate was also run with only the 25 pg/ml standard spike to provide a baseline and the data reported are corrected for this. Animals used for ELISA characterization of neurotrophin levels only received saline perfusion and were not post-fixed in paraformaldehyde. The brains were removed, snap frozen, and then allowed to equilibrate to -20°C . The brains were sectioned on a cryostat at -20 - 22°C at 400 μm thickness and the MS/vDB and hippocampi were dissected with a 0.75 mm micropunch on a -20°C freezing platform. The dissected tissue was weighed on a microgram scale, and then transferred to 25 μl of homogenizing solution (50 mM Na/Na₂ and 0.2% TX-100 in H₂O with Anti-protease Complete TM cocktail (Boehringer)) per mg of wet weight tissue. The tissue was sonicated in the homogenizing solution for 30-sec on ice

and centrifuged at 16,000 g for 15 min at 4⁰ C. The supernatant was collected and stored at -20⁰ C for ELISA analysis.

Levels of NGF and BDNF were analyzed in the homogenates from MS/vDB and hippocampus using a commercially available E_{max} Immunoassay System according to the manufacturer's instructions (Promega). Briefly, the 96-well plates were coated with 1:6,250 anti-NGF pAb in carbonate coating buffer (0.025 M sodium bicarbonate, 0.025 M sodium carbonate, pH 9.7) and incubated overnight at 4⁰ C. The plates were washed with TBST wash buffer (20 mM Tris-HCL pH 7.6, 150 mM NaCl, 0.05% (v/v) Tween 20) and blocked with 1x Block and Sample buffer (provided with kit) for 1 hour. The plates were washed again with TBST and a set of standard curves were generated in duplicate by performing 1:2 dilutions of a known 500 pg/ml standard in a range from 500 pg/ml to 7.6 pg/ml followed by a "blank" well of 0 pg/ml. All added samples and standards were allowed to incubate at 25⁰ C for 6 hours. The plates were washed thoroughly with TBST and 1:4000 anti-NGF mAb was added and incubated overnight at 4⁰ C. The plates were again washed with TBST and 1:100 anti-rat IgG pAb conjugated to HRP was added for 2.5 hours at room temperature. The plates were washed and TMB One Solution was added for color development for 10 min. The reaction was stopped with the addition of equal volume of 1 N HCl and the absorbance was read at 450 nm within 30 min of the color development reaction. The data were reported as pg of protein per mg wet weight tissue.

Statistical Analysis

Results are reported as the mean $\pm$ SEM. Statistical differences between groups were determined using analysis of variance (ANOVA).

Results

Cholinergic MS/vDB Cell Number in 21-day-old Mice and GABAergic Cell Number in Adult Mice

In 8-12-week-old mice, no significant differences were apparent in the relative number of stereologically counted Parv-positive neurons between groups ($F(1,10)=0.002$, $p=0.964$). Thus, the GABAergic neurons appear to be unaffected by IL-2 gene deletion. We did not assay for GABAergic alterations in younger animals, since there were no differences in the adult IL-2 KO mice relative to the wild-types.

In contrast to the previously reported data from 8-12-week-old animals (Beck et al., 2002), there was no significant difference in stereologically counted cholinergic somata number in the MS/vDB of 21-day-old IL-2 KO mice relative to wild-type mice ($F(1,12)=0.689$, $p=0.423$). As expected, the 21-day-old KO mice also did not exhibit splenomegaly seen in the autoimmune 8-12-week-old group, as spleen weights did not differ between 21-day-old wild type and IL-2 KO mice ($F(1,12)=0.989$, $p=0.340$).

Reduction in the IP-GCL Neuronal Number in IL-2 KO Mice

The IP-GCL of IL-2 KO mice had significantly fewer neuronal somata than wild-type mice (Fig. 3-1; $F(1,12)=10.966$, $p=0.006$). In the SP-GCL, however, there was no significant difference in granule cell number (Fig 3-1.; $F(1,12)=0.197$, $p=0.665$).

Alterations in Neurotrophin Levels

Levels of NGF protein in hippocampal tissue homogenates was significantly increased in IL-2 KO mice relative to wild-type mice (Fig. 3-2A; $F(1,10)=8.261$, $p=0.017$). The levels of BDNF protein, conversely, were significantly decreased in IL-2 KO mice relative to wild-type mice (Fig. 3-2B; $F(1,12)=8.023$, $p=0.015$).

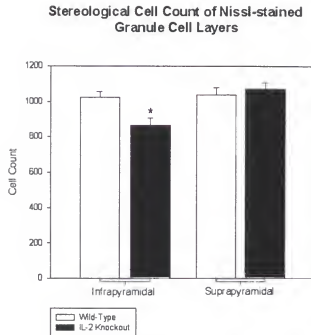
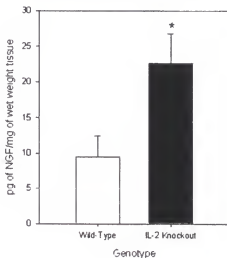


Fig. 3-1. There was a significant reduction in infrapyramidal, but not suprapyramidal granule cells in IL-2 knockout relative to wild-type mice. Each bar represents the mean $\pm$ SEM of seven animals per group. * $p=0.006$.

A) Enhanced NGF Levels in the Hippocampus of IL-2 Knockout Mice



B) Reduced BDNF Levels in the Hippocampus of IL-2 Knockout Mice

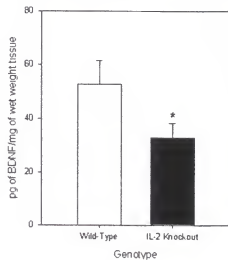


Figure 3-2. There was a significant: A) increase in NGF and B) decrease in BDNF protein levels in the hippocampus of IL-2 knockout compared to wild-type mice. The NGF bars represent the mean $\pm$ SEM of 7 animals per group. The BDNF bars represent mean $\pm$ SEM of 7 IL-2 knockout and 9 wild-type mice. * $p<0.05$.

Discussion

These data are the first to demonstrate that the loss of endogenous IL-2 in knockout mice can lead to alterations in neuronal cell number in the IP-GCL and production of the neurotrophins, BDNF and NGF. Further, this study expands upon the previous finding that IL-2 gene deletion leads to a deficiency of cholinergic neurons in the MS/vDB (Beck et al., 2002), by showing a lack of significant cholinergic neuronal differences in MS/vDB of 21-day-old IL-2 KO animals or GABAergic alterations in adult animals.

The lack of a difference in GABAergic neurons in 8-12-week-old adult mice was consistent with our initial hypothesis, as there is no evidence in the literature that IL-2 has any modulatory effects on GABAergic neurons. Moreover, this is not a regional effect, but rather appears to be selective to cholinergic projection neurons. As previously mentioned, IL-2 is a potent modulator of ACh release (Hanisch et al., 1993; Seto et al., 1997), and its precursor enzyme ChAT (Mennicken and Quirion, 1997), suggesting an effect of IL-2 on cholinergic neurons. In GABAergic neurons, however, IL-2 has failed to evoke release of GABA in mesencephalic neuronal cultures (Alonso et al., 1993), or the cortex or hippocampus of mice (Bianchi et al., 1995). Since IL-2 deficiency does not affect the number of GABAergic somata in the MS/vDB of IL-2 KO mice, the neuronal loss appears to be selective for cholinergic neurons in the MS/vDB. Furthermore, we previously found no differences in the striatal cholinergic neuronal number (Beck et al., 2002), so the lack of IL-2 does not simply cause a general loss of all cholinergic neurons.

Against our initial hypothesis that 21-day-old IL-2 KO mice would have similar cholinergic deficiencies as adult 8-12-week-old mice, there was no detectable loss of cholinergic cell number in the MS/vDB. We did not examine 21-day-old mice for

differences in GABAergic cell number, since we did not detect any changes in adult mice using the same marker. One potential explanation for the loss of cholinergic neurons in the MS/vDB may be a failure in maintenance or survival in the late stages of, or after, development. Other studies have found decreases in cholinergic enzyme activity (i.e., ChAT and AChE) between postnatal days 30-60 in normal rats (Thal et al., 1992), and postnatal days 60-150 in C57BL/6 mice (Virgili et al., 1991). The IL-2 KO mice may potentially be more susceptible to this loss of cholinergic activity during adulthood, which could lead to the previously observed deficiencies in 8-12-week-old IL-2 KO animals.

An alternate explanation for the different cholinergic effects seen in 21-day-old vs. 8-12-week-old animals is that the loss of IL-2 may be secondary to the effects of autoimmunity present in adult IL-2 KO animals. Though we cannot completely rule out this possibility, we have previously failed to find discernable levels of infiltrating lymphocytes or clear signs of gliosis in the brains of IL-2 KO animals (Petitto et al., 1999). More research is necessary to further address this issue.

Another finding of this study was a significant decrease in neuronal cell number in the IP-GCL, but not the SP-GCL. This decrease is consistent with the *in vitro* studies showing a potent neurotrophic effect of IL-2 on hippocampal neurons (Awatsuji et al., 1993; Sarder et al., 1996; Sarder et al., 1993). Furthermore, these data are supported by our previous findings that IL-2 KO mice exhibited a reduced distance across the IP-GCL (Beck et al., 2002), and that the IP mossy fiber length of IL-2 KO mice is shorter than wild-type controls (Petitto et al., 1999). The reductions in distance across the IP-GCL could also potentially be explained by increased density, but not number, of cells or

smaller cell body size. Qualitative assessments of random granule cells, however, do not support this hypothesis, though a more extensive study would be necessary to definitively address that issue.

Though the receptors for IL-2 are more abundant in the hippocampus, including the GCL of the DG (Petitto and Huang, 1994; Petitto et al., 1998), it is not clear whether IL-2 may act directly on these neurons, or whether it upregulates other growth factors like the neurotrophins. The observed differences in the level of the neurotrophin BDNF was consistent with our hypothesis that we would find a reduction in trophic factors important in MS/vDB and hippocampal development and maintenance. BDNF plays a role in the maintenance and repair of septal cholinergic neurons (Alderson et al., 1990; Morse et al., 1993; Ward and Hagg, 2000), can implement a positive feedback mechanism with these neurons to enhance the release of ACh (Knipper et al., 1994), and can also modulate neurogenesis (Larsson et al., 2002; Lee et al., 2002), thus potentially impacting granule cell number. Thus, the reduction of cholinergic cell number in the MS/vDB is consistent with a reduction in this trophic factor. The exact interaction between IL-2 gene deletion and the reduction of BDNF levels remains unclear. Though BDNF is expressed in the peripheral immune system by lymphocytes, IL-2 does not stimulate its production or release. IL-2 can, however, upregulate the expression of TrkB, the receptor for BDNF, in lymphocytes (Besser and Wank, 1999). Furthermore, some evidence suggests that BDNF can stimulate a positive feedback mechanism of its own production via the TrkB receptor in hippocampal neurons (Canossa et al., 1997; Saarelainen et al., 2001). In IL-2 KO mice, the absence of IL-2 may therefore potentially lead to a down-regulation of the TrkB receptor, thereby partially inhibiting the positive

feedback production of BDNF. Interestingly, the neurotrophin Trk receptors and IL-2 receptor share some of the same signal transduction pathways (e.g., mitogen activated protein kinase or phosphatidylinositol 3-kinase), which appear to play a role in their growth and survival promoting actions (for reviews see Gaffen, 2001; Patapoutian and Reichardt, 2001). Whether IL-2 knockout leads to disruption of one of these signal transduction pathways has not, to our knowledge, been elucidated and thus requires further study.

Against our initial hypothesis, NGF protein levels were actually increased in the IL-2 KO mice. Unlike BDNF, NGF does not appear to stimulate a positive feedback neurotrophin release from hippocampal neurons (Canossa et al., 1997). Given the reduction in cholinergic survival in the MS/vDB of IL-2 KO mice, the target neurons in the hippocampus of these animals may produce higher protein levels of NGF as a compensatory response. Similarly, moderate lesions of rat septohippocampal projections lead to increased mRNA expression of NGF, but not BDNF in hippocampal target cells (Hellweg et al., 1997).

In summary, cholinergic deficits seen in the MS/vDB of IL-2 KO mice appear to be selective for cholinergic over GABAergic neurons. In addition, the loss of cholinergic neurons in the MS/vDB may occur in the later stages of, or after, development of the septohippocampal system, as the deficits are not seen in 21-day-old IL-2 KO mice. In the hippocampus, the number of neurons in the IP-GCL is significantly reduced. A reduced production of hippocampal BDNF may contribute to many of the aforementioned changes, though NGF levels are increased in a possible compensatory response. Although overt signs of autoimmunity in the brain are not apparent (we have been unable

to detect significant levels of leukocyte infiltration or gliosis in IL-2 KO mice brains), further study is necessary to assess this possibility, as factors such as IL-2 induced cytokine dysregulation or autoantibodies could contribute to the hippocampal alterations in adult IL-2 KO mice.

CHAPTER 4

INTERLEUKIN-2 DEFICIENCY: NEUROIMMUNOLOGICAL STATUS AND NEUROGENESIS IN THE HIPPOCAMPUS

Introduction

Receptors for interleukin-2 (IL-2) are enriched in the hippocampal formation, and many of the most prominent neurobiological functions of this cytokine occur in the hippocampus (Araujo et al., 1989; Hanisch and Quirion, 1995a; Lapchak et al., 1991; Petitto and Huang, 1994, 2001; Petitto et al., 1998). Previous studies from our laboratory have found that IL-2 knockout (KO) mice exhibit significantly lower numbers of medial septum and vertical limb of the diagonal band of Broca (MS/vDB) cholinergic cell bodies, a reduction in the distance across the granular cell layer (GCL) of the infrapyramidal (IP) blade of the dentate gyrus (DG), and decreased fiber length and neuronal cell number in the IP-GCL of the DG (Beck et al., 2004; Beck et al., 2002; Petitto et al., 1999). These neurobiological alterations appear to be related to abnormalities in learning and memory performance and sensory motor gating in IL-2 KO mice (Cushman et al., 2004; Petitto et al., 1999).

Because IL-2 has been shown to possess various neurotrophic and neuromodulatory effects on hippocampal neurons *in vitro* (Awatsuji et al., 1993; Bianchi et al., 1995; Pauli et al., 1998; Plata-Salman and French-Mullen, 1993; Sarder et al., 1996; Sarder et al., 1993; Tancredi et al., 1990), our original working hypothesis was that the alterations exhibited by IL-2 KO mice are due to the absence of IL-2's neurotrophic actions on hippocampal neurons during development. More recent data from our

laboratory, however, suggests that these hippocampal changes may be due to neurodegenerative rather than neurodevelopmental processes. We tested mice at postnatal day 21 (P21), an age where septohippocampal cholinergic neurons are nearly fully developed, to determine if the reduction in septohippocampal cholinergic projection neurons seen in adult IL-2 KO mice was present earlier in postnatal development (e.g., at weaning) and prior to the onset of the earliest signs of autoimmune disease (e.g., splenomegaly, lymphadenopathy). Contrary to our hypothesis, we found that the number of MS/vDB cholinergic cell bodies did not differ between IL-2 KO and wild-type littermates at P21 (Beck et al., 2004). Thus, together these data indicate that the loss of cholinergic neurons that occurs between P21 and adulthood (8-12 weeks) suggests an alternate hypothesis; neurodegenerative processes may be operative in the brain of IL-2 KO mice.

Since IL-2 is an important factor in immune physiology, one possible mechanism behind these neurodegenerative processes is immune dysregulation caused by the absence of IL-2. IL-2-deficiency in mice leads to generalized systemic autoimmune disease in adult mice that may affect multiple organs in the periphery, most notably the intestines and the kidneys (Horak, 1995). The autoimmune effects in IL-2 KO mice involving peripheral organs are mediated largely by infiltrating T cells. In the colon, for example, adult IL-2 KO mice develop chronic inflammatory bowel disease with features common to inflammatory ulcerative colitis in humans, where the lamina propria is infiltrated with activated T cells responsible for the development of this inflammatory disease (Ma et al., 1995). In addition, there is a disruption of immune homeostasis that is evidenced by changes in the gene expression of several Th1, Th2, and various proinflammatory

cytokines in this organ (Autenrieth et al., 1997; Meijssen et al., 1998). Moreover, these cytokine changes and the onset of inflammatory bowel disease are preceded by increased gene expression of IL-15, which shares the same signal transducing receptor subunits with IL-2 (Meijssen et al., 1998). Thus, it is possible that the immune dysregulation in the brain of IL-2 KO mice may be induced by activated T cells and/or proinflammatory cytokines (e.g., IL-1, TNF α , IL-6) from the periphery crossing the blood-brain-barrier (BBB).

By contrast, IL-2 may lead to neuroimmunological changes that do not involve peripheral immune cells. Rather than peripheral T cells and serum cytokines entering the brain, an alternative hypothesis that may account for the hippocampal differences observed in P21 versus adult IL-2 KO mice may be that the absence of IL-2 reduces the trophic support of hippocampal neurons as a result of dysregulation of other brain-derived cytokines. Thus, loss of IL-2 in the brain could in turn modify the normal neuroimmunological status of the brain by modifying the normal expression of brain cytokines such as IL-15. Since IL-2 can modify the release of certain cytokines from lymphoid cells (Lauwerys et al., 2000; McDyer et al., 2002), similar actions could occur in brain cells that produce cytokines (e.g., microglia, astrocytes). Alterations in the production of brain cytokines important in normal brain physiology could alter the integrity of hippocampal neurons by decreasing levels of classic neurotrophins and/or neurotrophic cytokines on the one hand, or elicit inflammatory-like neurodegenerative processes within the brain on the other.

The present study therefore sought to test the hypothesis that IL-2 gene deletion results in neuroimmunological changes in the hippocampus by examining the possible

outcomes described above. We compared the hippocampi of adult IL-2 KO mice and wild-type littermates at 8-12 weeks of age, the age where differences in hippocampal cytoarchitecture and behavior have been found previously (Beck et al., 2004; Beck et al., 2002; Cushman et al., 2004; Petitto et al., 1999), for differences in several measures of neuroimmunological status. First, the groups were assessed for differences in the number of CD3⁺ T lymphocytes and activated microglial cells (as measured by MHC-II positivity) in the hippocampus. IL-15 is also expressed in the brain (Hanisch et al., 1997a; Lee et al., 1996), and this cytokine is known to have both proinflammatory and anti-inflammatory effects, potent anti-apoptotic, and T cell chemoattractant properties (Wilkinson and Liew, 1995). Because IL-15 uses the IL-2/15R β and γ_c subunits that are enriched in the neuronal cell layers of the hippocampus (Petitto and Huang, 2001), and may modulate microglial cell function and T cell chemoattraction, a second aim of this study was to test the hypothesis that IL-15 is elevated in the hippocampus of IL-2 KO mice. In addition, since changes in both IL-2 and IL-15 may modify levels of various cytokines in other tissues and physiological contexts, exploratory testing was performed to determine if IL-2 KO mice have increased levels of proinflammatory cytokines in the hippocampus relative to wild-type mice (also, compared to serum levels). Furthermore, as recent evidence indicates that elevation of inflammatory cytokines such as IL-6 may impair hippocampal neurogenesis (Monje et al., 2003; Vallieres et al., 2002), a third aim of this study was to test the hypothesis that the postulated changes in neuroimmunological status would be associated with reductions in neurogenesis of neurons in the dentate gyrus (DG) of IL-2 KO mice.

Materials and Methods

Animals and Genotyping

Mice used in these experiments were cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Mice were bred in our colony using IL-2 heterozygote by heterozygote crosses. The IL-2 KO mice, obtained originally from the NIH repository at Jackson Laboratories, were derived from ten generations of backcrossing onto the C57BL/6 background. Mice were housed under specific pathogen-free conditions. Animals used in these experiments were 8-12 weeks of age. Independent animals were used for the assessment of CD3⁺ T cells and MHC II⁺ microglial cells in the hippocampus, the determinations of hippocampal versus serum cytokine levels, and assessments of neurogenesis in the dentate gyrus. Specific numbers utilized are reported at the beginning of the description of each method.

The genotypes of all mice were determined by the polymerase chain reaction (PCR). PCR reactions were performed using 25 µl total reaction volume containing 1 µM each of forward and reverse primers, 0.1 µg genomic DNA, 0.2 mM of each dNTP, 0.3 µl Taq DNA polymerase, and amplified using a thermal cycler with a heated evaporation cover (Ericomp). The cycling parameters were hot start 95°C (3min), denaturing 94°C (30 sec), annealing 64°C (30 sec), extension 72°C (45 sec) with a final extension step of 4 min. Thirty cycles were used for these experiments. The 5' and 3' primers for the IL-2 KO (500 bp knockout band amplified) were 5'-TCGAATCGCCAATGACAAGACGCT-3' and 5'-GTAGGTGGAAATTCTAGCATCATCC-3'. The 5' and 3' primers for the IL-2 wild type (324 bp wild type band amplified) were 5'-

CTAGGCCACAGAATTGAAAGATCT-3' and 5'-
GTAGGTGGAAAATTCTAGCATCATCC-3'.

CD3⁺ T cells and MHC II⁺ Microglia Immunohistochemistry

For qualitative assessment of autoimmunity, three IL-2 KO and three wild-type brains were processed for MHC II (an activated microglial marker) and CD3 (a pan T cell marker) immunohistochemistry. Each animal was anesthetized with an injection cocktail of 3:3:1 ketamine (100 mg/ml): xylazine (20 mg/ml): acepromazine (10 mg/ml) at a dose of 0.015 ml injection cocktail/g body weight and perfused with 0.9% saline followed by 4% paraformaldehyde in phosphate buffered saline (PBS). The brains and spleens were removed and fixed overnight in 4% paraformaldehyde, followed by overnight equilibration in 30% sucrose cryoprotective solution, and then were snap frozen in isopentane (-80°C) for storage. The spleens were weighed to assay for relative splenomegaly of IL-2 KO vs. wild-type mice. The brains were equilibrated to -20°C prior to cryostat sectioning into 50 µm slices in the coronal plane, collected into individual wells of polystyrene 24-well plates (NUNC 1147), and stored free-floating at 4°C in PBS for histochemistry. Every third section was processed for MHC II or CD3 immunohistochemistry.

Free-floating 50-µm sections were incubated for 20 minutes in 1% hydrogen peroxide (H₂O₂) to quench endogenous peroxidative activity. The sections were then washed and blocked for 1 hr in 200 µl/well 3% normal goat serum (NGS). After this incubation, the sections were incubated overnight in the primary antibody, rat anti mouse CD3 (BD PharMingen; 1:500 in PBS with 0.3% Triton X-100 and 1% NGS) or rat anti mouse MHC II (BD PharMingen; 1:500 in PBS with 0.3% Triton X-100 and 1% NGS). The next day, the sections were washed and incubated overnight in the secondary

antibody, biotinylated goat anti-rabbit IgG (Sigma B-7389; 1:1000 dilution in PBS with 0.3% TX-100 and 1% NGS). The sections were then washed and incubated in ExtrAvidin (Sigma E-2886; 1:1000 in PBS) for 2 hrs. The sections were developed in 0.5 mg/ml 3,3'-diaminobenzidine (DAB), 0.2 mg/ml urea H₂O₂ for approximately 5 min and were placed on slides, dehydrated in graded ethanol washes, cleared in two changes of xylenes, and coverslipped.

Preparation of Serum and Brain Tissue for Cytokine Analysis

Hippocampal homogenates were analyzed from eight IL-2 KO and nine wild-type mice to measure cytokine levels in the hippocampus. From these subject groups, serum was collected from a smaller subset of animals (five IL-2 KO and seven wild-type mice) for comparative analysis of brain vs. peripheral cytokine levels. Animals used for characterization of endogenous cytokine levels were anesthetized with an injection cocktail of 3:3:1 ketamine (100 mg/ml): xylazine (20 mg/ml): acepromazine (10 mg/ml) at a dose of 0.015 ml injection cocktail/g body weight. Whole blood was collected by puncturing the right atrium of the heart and inserting heparinized micro-hematocrit capillary tubes (Fisher Scientific). The animals were then saline perfused, but were not post-fixed in paraformaldehyde. The whole blood was centrifuged in Microtainer Brand serum separator tubes (Becton Dickinson) at 5,000 rpm for 10 minutes to isolate serum and the serum was stored at -80°C until used for Luminex analysis. The brains were removed, snap frozen, and then allowed to equilibrate to -20°C . The brains were sectioned on a cryostat at -20 – -22°C at 400 μm thickness and the hippocampi were dissected with a 0.75 mm micropunch on a -20°C freezing platform. The dissected tissue was weighed on a microgram scale, and then transferred to 25 μl of homogenizing

solution (50 mM Na/Na₂ and 0.2% TX-100 in H₂O with Anti-protease Complete TM cocktail (Boehringer)) per mg of wet weight tissue. The tissue was sonicated in the homogenizing solution for 30 sec on ice and centrifuged at 16,000 g for 15 min at 4⁰ C. The supernatant was collected and stored at -20⁰ C for Luminex analysis.

Multiplex Microsphere Cytokine Analysis

Commercial kits, Lincoplex mouse cytokine (Linco, Research, Inc) and a Luminex 100 LabMAP system (Upstate Biotechnology), were used in attempt to measure a number of cytokines in the hippocampus and in the serum of IL-2 KO and wild-type mice. Assays were performed according to the manufacturer's instructions, and cytokine concentrations were calculated using the Softmax program and the linear range on the standard curve (3.2–10,000 pg/ml). Altogether, we attempted to detect a total of twenty-two different cytokines and chemokines from the serum and brain homogenates of these animals. In the serum, there were detectable levels of IL-6, IL-13, keratinocyte-derived chemokines (KC), granulocyte-colony stimulating factor (G-CSF), and macrophage inflammatory protein-1 alpha (MIP-1 α). In the brain, there were detectable levels of IL-7, IL-9, IL-12, IL-15, interferon-gamma inducible protein of 10 kD (IP-10), and monocyte chemoattractant protein-1 (MCP-1). Thus, only those cytokines and chemokines detected were subjected to statistical analyses. The remainder of the cytokines and chemokines tested could not be detected in either the serum or the brain (IFN- γ ; TNF- α ; IL-1 α , IL-1 β ; IL-2, IL-4, IL-5, IL-10, IL-17, GM-CSF, and RANTES).

Labeling Neurogenesis with BrdU

Twelve IL-2 KO (six male; six female) and eleven wild-type (five male; six female) mice were used to assay for differences in neurogenesis. The procedure for

labeling of neurogenesis and subsequent immunostaining in the mouse hippocampus has been adapted from (Lee et al., 2002). Briefly, the mice were given five intraperitoneal injections of BrdU (50 mg/kg of body weight) over the course of 3 days. The day following the last injection, the mice were sacrificed and perfused with 0.9% saline followed by 4% paraformaldehyde in PBS as described previously.

The BrdU-incorporated brains were equilibrated to -20°C and cryostat-sectioned at $50\text{ }\mu\text{m}$ in the coronal plane. They were collected into individual wells of polystyrene 24-well plates (NUNC 1147), and used for free-floating immunohistochemistry. The sections were then washed twice in PBS and then the DNA was denatured by a 30 minute incubation with 2 N HCl to allow binding of the antibody to the BrdU in the single-stranded DNA. The acid was neutralized with a 0.1 M borate buffer (pH 8.5) wash, followed by several washes in PBS. Afterwards, the sections were blocked for 1 hr in 3% normal goat serum (NGS). The sections were then incubated overnight in the primary antibodies, rat monoclonal anti-BrdU (Serotec; 1:400 in PBS with 0.3% Triton X-100 and 1% NGS) and either the neuronal marker mouse monoclonal anti-tubulin β III isoform (Chemicon; 1:200 in PBS with 0.3% Triton X-100 and 1% NGS), the astroglial marker rabbit anti-glial fibrillary acidic protein (GFAP; Chemicon; 1:1,000 in PBS with 0.3% Triton X-100 and 1% NGS) or the oligodendrocyte marker mouse anti-2'3'-cyclic nucleotide 3'-phosphohydrolase (CNPase; Chemicon; 1:200 in PBS with 0.3% Triton X-100 and 1% NGS). The next day, the sections were washed twice in PBS and incubated for 2 hr in the dark with the secondary antibodies, goat anti-rat IgG (H+L) conjugated with Alexa Fluor-488 (green; Molecular Probes; 1:400 in PBS with 0.3% TX-100 and 1% NGS) and goat anti-mouse IgG (highly cross-absorbed H+L) conjugated with Alexa

Fluor 568 (red; Molecular Probes; 1:400 in PBS with 0.3% TX-100 and 1% NGS) or goat anti-rabbit IgG (H+L) conjugated with Alexa Fluor 350 (blue; Molecular Probes; 1:400 in PBS with 0.3% TX-100 and 1% NGS). The sections were then washed twice in PBS, placed on slides, dehydrated in graded ethanol washes, cleared in two changes of xylenes, and coverslipped.

The sections were imaged using a Bio-Rad 1024 ES confocal microscope and only cells which showed colocalized staining through five consecutive 1- μ m planes were considered to be double-labeled. The IP and SP-GCL area (mm^2) were measured at 20x magnification using the MCID 5.1 software, a CCD High Resolution Sony camera, and a Zeiss Axioplan 2 microscope. The data were reported as a density of all double-labeled cells counted from five sections per animal divided by the total area measured

Results

Assessment of CD3⁺ T Cells and MHC II⁺ Activated Microglia in the Hippocampus

No CD3⁺ T cells were detected in the hippocampi of either 8-12-week-old IL-2 KO or wild-type mouse brains, and only an occasional MHC II⁺ microglial cell was detected (e.g., approximately one every other section) in both groups. By contrast, both activated MHC II⁺ microglia and CD3⁺ T cells were readily detectable in positive control slices (sections of the axotomized FMN of wild-type C57BL/6 mice; Petitto et al., 2003) demonstrating that the immunohistochemistry procedure utilized was effective for labeling both markers. Thus, no differences in T cells or activated microglial cells were found between the subject groups.

Hippocampal Cytokine Levels in IL-2 Knockout vs. Wild-type Mice

As depicted in Figure 4-1, in the hippocampus, there were significantly increased levels of IL-12 (increased ~57%; $F(1,15)=9.174$, $p=0.008$) and IL-15 (increased ~38%;

$F(1,15)=6.105$, $p=0.026$) in the IL-2 KO compared to wild-type mice. Figure 4-1 also shows that IL-2 KO mice had increased levels of the chemokines, IP-10 (increased ~63%; $F(1,15)=4.747$, $p=0.046$) and MCP-1 (increased ~46%; $F(1,15)=5.218$, $p=0.039$). Although detectable levels of IL-7 and IL-9 were found in hippocampus, they did not differ between the subject groups.

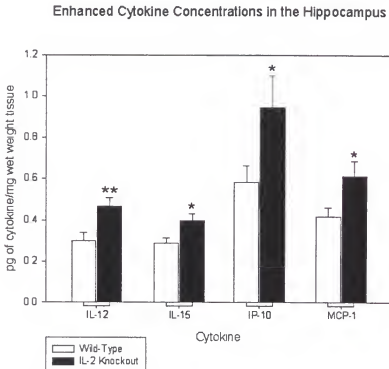


Figure 4-1. Protein levels of several cytokines and chemokines are elevated in the IL-2 knockout mice compared to wild-type brain. These include IL-12, IL-15, IP-10, and MCP-1. * $p<0.05$; ** $p<0.01$.

Comparison of Serum Cytokine Levels in IL-2 Knockout vs. Wild-type Mice

As can be seen in Figure 4-2, the IL-2 KO mice exhibited marked elevation in serum IL-6 (increased ~224%; $F(1,10)=8.077$, $p=0.017$), and serum MIP-1 α serum concentration (increased ~327%; $F(1,10)=21.538$, $p=0.001$) compared to wild-types. Although detectable levels of IL-13, G-CSF, and KC were detectable in the serum, levels of these cytokines did not differ between the subject groups.

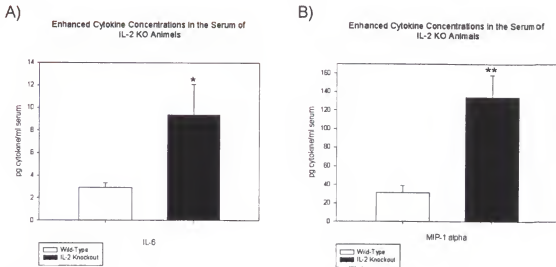


Figure 4-2. Relative cytokine profile of IL-2 knockout mouse serum does not match brain profile. A) IL-6 is increased in IL-2 knockout mice compared to wild-type; and B) the chemokine MIP-1 α is higher in IL-2 knockout mice than wild-type. * $p < 0.05$; ** $p < 0.01$.

Alterations in Neurogenesis

Although there was not a significant effect of group on neurogenesis in either the IP-GCL or SP-GCL, as depicted in Figure 4-3 and 4-4 respectively, there was a significant group by gender interaction in both the IP-GCL ($F(1,19)=4.71$, $p=0.043$) and SP-GCL ($F(1,19)=6.43$, $p=0.02$). Fisher's least significant difference post hoc analysis test confirmed a difference between male IL-2 KO and wild-type mice in the IP-GCL ($p=0.025$) and SP-GCL ($p=0.014$), but not between female groups in either region. There was no significant effect of group or group by gender interaction in cells around either the IP-GCL or SP-GCL labeled with the oligodendrocyte marker, CNPase (data not shown). Similarly, no significant effect of group or group by gender interaction was evident in either the IP or SP-GCL in cells labeled with the astrocyte marker, GFAP (data not shown).

Neurogenesis in the Infrapyramidal Granule Cell Layer

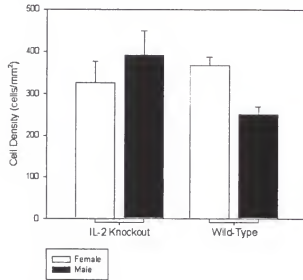


Figure 4-3. There is no effect of group on neurogenesis in the infrapyramidal granule cell layer, but there is a group by gender interaction between IL-2 knockout and wild-type mice ($p=0.043$). This effect appears to be attributable to differences in the male mice (Fisher least significant difference test, $p=0.025$).

Neurogenesis in the Suprapyramidal Granule Cell Layer

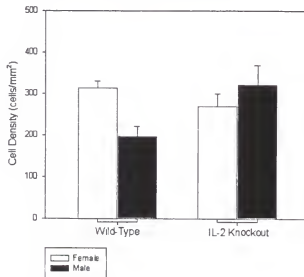


Figure 4-4. There is no effect of group on neurogenesis in the suprapyramidal granule cell layer, but there is a group by gender interaction between IL-2 knockout and wild-type mice ($p=0.02$). This effect appears to be attributable to differences in the male mice (Fisher least significant difference test, $p=0.014$).

Discussion

The data presented here show that IL-2 KO and wild-type littermates exhibit differences in several measures of neuroimmunological status in the hippocampus. In order to access the brain parenchyma, T cells require activation markers to cross the BBB (Hickey et al., 1991). We have previously reported leukocytes were not detectable in the cresyl violet stained hippocampal sections from IL-2 KO mice (Petitto et al., 1999), however, we recognized that it is difficult to reliably detect small numbers of peripheral leukocytes in the brain without cell-type specific stains. Although we were therefore not expecting to see substantial numbers of T cells in the IL-2 KO brain, we wanted to determine if small numbers of autoimmune T lymphocytes were present that could initiate neuroimmunological alterations in the hippocampus. The hippocampi of IL-2 KO mice were devoid of T cells, despite the fact that the majority of peripheral T cells of IL-2 KO mice express activation markers such as CD69 (Sakai et al., 1995; Schopke et al., 1991), which are thought to enhance their ability to cross the BBB. Microglia are indigenous antigen presenting cells (Hickey and Kimura, 1988; Streit et al., 1988). Contact with T cells can induce microglia to exhibit characteristics of antigen presenting cells, and microglia also have the ability to activate T cells (Aloisi et al., 2000). There was, however, no evidence of increased numbers of activated microglia in the hippocampus of IL-2 KO mice. This observation is consistent with our previous finding in C57BL/6*scid*-IL-2 KO (mice without mature T and B cells), which were devoid of T cells in the axotomized facial motor nucleus and had levels of axotomy-induced activated microglia that did not differ from wild-type mice (Petitto et al., 2003). Thus, at the cellular level, the hippocampus of IL-2 KO mice did not show signs of autoimmune disease.

Cytokines can enter brain via specific transport mechanisms and through the circumventricular organs. In this study, we measured levels of the various cytokines of interest in the serum to determine if levels found in the hippocampus correlated with those found in the serum. If a particular inflammatory cytokine (e.g., TNF α) had been found to be significantly elevated in the serum and the hippocampus of IL-2 KO mice, it would have suggested the possibility that peripheral immune activation associated with autoimmunity (Schimpl et al., 2002) could account for the presence of the cytokine in the hippocampus (although increased gene transcription and translation in both the periphery and the brain could not be ruled out). Although there were significantly increased levels of IL-6 and MIP1 α in the circulation of IL-2 KO mice, we did not detect either of these proteins in the brain. IL-6 is a proinflammatory cytokine that was of particular interest to us because of its actions in the hippocampus, including effects on neurogenesis (Monje et al., 2003; Vallieres et al., 2002). Although levels of IL-6 were markedly increased in the serum, though not measurable in the hippocampus at the tissue homogenate concentrations used, the unlikely possibility remains that IL-6 could have entered the brain from the circulation and had functional consequences at concentrations below the limits of detection of the assay method. Nonetheless, the most parsimonious explanation is that cytokines from the peripheral circulation of IL-2 KO mice are not the source of the cytokine alterations found in the hippocampus. Thus, peripheral cytokine dysregulation associated with autoimmunity in IL-2 KO does not appear to be associated with their hippocampal pathology.

Our data indicate that loss of IL-2 in the brain results in changes in the production of several other brain cytokines. Consistent with our hypothesis, IL-15 concentrations

were increased in the hippocampus of IL-2 KO mice. IL-15 is structurally related to IL-2 and shares the same β and γ_c signal transducing receptor subunits with the IL-2 receptor (Giri et al., 1995). IL-15 also shares and opposes several physiological functions of IL-2 in the peripheral immune system (Waldmann, 2002; Waldmann et al., 2001). IL-15 and its heterotrimeric receptor are constitutively expressed in various regions of the adult mouse brain and can be detected in microglial cultures (Hanisch et al., 1997a), astrocytes (Lee et al., 1996), and possibly neurons (Maslinska, 2001; Satoh et al., 1998). As noted earlier, increased IL-15 gene expression precedes the inflammatory cytokine changes and onset of inflammatory bowel disease in IL-2 KO mice (Meijssen et al., 1998). It also induces the onset of autoimmunity in thyroiditis (Kaiser et al., 2002). Thus, IL-15 could trigger proinflammatory cytokine-like processes in the hippocampus, including the elevations in IL-12 that were found in IL-2 KO mice in this study. IL-12-driven Th1 responses are involved in inflammation (e.g., colonic) in IL-2 KO mice (Ludviksson et al., 1997), and it has been implicated as an important effector in the pathogenesis of experimental autoimmune encephalomyelitis (EAE) (Adorini, 1999; Segal et al., 1998). Moreover, IL-15 can render cells resistant to the protective effects of TGF β (Campbell et al., 2001), a Th2 cytokine that appears to play a key role in dampening processes associated with peripheral autoimmune disease in IL-2 KO mice (Ludviksson et al., 1997). Thus, these actions of IL-15 suggest that it may be involved in the hippocampal pathology seen in IL-2 KO mice. It is noteworthy, however, that IL-15 has potent anti-apoptotic properties (Lauwerys et al., 2000; Waldmann, 2002; Waldmann et al., 2001) that may oppose the pro-apoptotic effects of IL-2. We have recently found that loss of brain IL-2 in C57BL/6scid-IL-2 KO mice increased neuroregeneration in the axotomized

facial motor nucleus (Petitto et al., 2003). It is interesting to speculate that elevated IL-15 levels could contribute to the increased motor neuronal survival in those mice. Therefore an alternative interpretation is that increased IL-15 in the hippocampus of IL-2 KO mice could be a compensatory response to counteract neuroregenerative changes in the hippocampus.

The increased levels of MCP-1 and IP-10 may possibly be induced by increased IL-15 in the hippocampus (Badolato et al., 1997). Previous studies have demonstrated that IP-10 expression can be detected in lipopolysaccharide (LPS)-treated microglial and astroglial cultures and in situ hybridization of LPS-treated rat brains (Ren et al., 1998). Similarly, MCP-1 can also be induced by the addition of the pro-inflammatory cytokine, TNF- α , or the anti-inflammatory cytokine, TGF- β , in astrocytes (Hurwitz et al., 1995) and microglia (Meda et al., 1996). IP-10 and MCP-1 are also chemoattractant factors for T lymphocyte infiltration into the CNS (Babcock et al., 2003; Dufour et al., 2002), and may attract activated microglia in vitro (Cross and Woodroffe, 1999). In spite of the published data linking IP-10 and MCP-1 to T cell and microglial chemotaxis, we were unable to detect either T cells or increased numbers of activated microglia in the hippocampus of IL-2 KO mice. Further studies are necessary to determine the functional significance of the increased production of IP-10 and/or MCP-1 in the hippocampus of IL-2 KO mice. Finally, in addition to its immune activating effects as a Th1 cytokine, IL-2 is also known to have important critical negative regulatory functions by stimulating Th2 lymphocytes to produce TGF β (Ludviksson et al., 1997), which down-regulates the ability of antigen presenting cells to produce IL-12, a powerful activator of Th1 cell

development and inflammation. Thus, the increased levels of IL-12 found in the hippocampus could be the secondary to the loss IL-2.

Increased levels of inflammatory cytokines such as IL-6 may impair hippocampal neurogenesis (Monje et al., 2003; Vallieres et al., 2002); thus, we hypothesized that the alterations in neuroimmunological status would correlate with decreased neurogenesis in the DG of IL-2 KO mice. Against our hypothesis, however, no detectable levels pro-inflammatory cytokines like IL-6 (though IL-12 can mediate inflammatory responses) or effects of group were apparent in hippocampal neurogenesis. However, a significant group by gender interaction was detectable. Interestingly, this effect appeared to be due to variations in the male mice, but not the females. We previously noted a trend which suggested that IL-2 gene deletion may protect from experimental autoimmune encephalomyelitis (EAE), though we did not statistically analyze the data (Petitto et al., 2000). In that study, three out of the seven male mice utilized developed some symptoms of the disease, whereas none of the six females did. One hypothesis to explain this group by gender interaction is that there may be an interplay between IL-2 and the sex hormones. Sex steroids like estrogen, for example, have been linked to neurogenesis (Gould et al., 2000; Perez-Martin et al., 2003). Further, evidence that IL-2 can regulate sex hormone expression comes from studies of Leydig cell cultures where IL-2 was shown to inhibit steroidogenesis (Guo et al., 1990).

Hormonal differences influenced by IL-2 may give a possible explanation of why males differ from females, but it does not suggest a mechanism of why IL-2 KO mice appear to be protected from impaired hippocampal neurogenesis. As noted above, absence of IL-2 can enhance neuroregenerative properties in the axotomized facial motor

nucleus and we speculate an involvement of IL-15. Though IL-15 has not been linked to neurogenesis, its antiapoptotic properties on immune cells are well-studied (Lauwerys et al., 2000; Waldmann, 2002; Waldmann et al., 2001). If these antiapoptotic actions of IL-15 can also promote survival of neurons, then this may provide a hypothesis of why the IL-2 KO male mice have higher levels of neurogenesis than the wild-types. Some cytokines are capable of promoting the development of neural stem cells (Rozenal et al., 1995; Shah et al., 1996; Wong et al., 2004), and this may account for the how they influence neurogenesis. The actual mechanism whereby IL-2, sex hormones, and neurogenesis may interact is as of yet undefined and requires future study.

In summary, T cells and peripheral cytokines do not appear to enter into the hippocampi of IL-2 KO mice, which does not support the hypothesis that the CNS alterations previously seen in IL-2 KO mice are due to peripheral autoimmunity. Other potential immune indicators of autoimmunity (e.g., deposition of autoantibodies in the brain) were not addressed in this dissertation and require more research. Genetic deletion of IL-2 may, however, alter the neuroimmunological status of the mouse hippocampus through a dysregulation of cytokines produced by CNS cells (e.g., microglia, astroglia). Further studies will be required to determine how these changes impact hippocampal cytoarchitecture and function in IL-2 KO mice.

CHAPTER 5 GENERAL DISCUSSION

Summary of the Overall Findings

The overall purpose of this dissertation research was to investigate the impact of IL-2 on the septohippocampal system by observing changes in the basal physiology and structure of IL-2 KO mice brains vs. their wild-type littermates. During these experiments, we chose not to surgically, chemically, or in any other way experimentally manipulate these animals beyond the genetic knockout of IL-2, so that the effects that we detected could be attributed to loss of IL-2 and not to confounding experimental techniques (e.g., disruption of the BBB). We understand, however, that some experimental manipulations could be useful in the long term and discuss the topic more in the “Caveats and Future Directions” section of towards the end of this chapter.

In the first study in Chapter 2, we determined that IL-2 KO mice suffered a significant 26% loss of cholinergic neurons in the MS/vDB relative to wild-type littermates. This loss of cholinergic somata was not reflected by a similar loss of cholinergic fiber density in the hippocampal projection fields of the septal cholinergic neurons. Moreover, the deficits observed were not a general effect on all cholinergic neurons of the brain, as the cholinergic neurons of the striatum in the IL-2 KO animals studied did not appear to be affected. There was, however, a reduction in the distance across the IP, but not SP, GCL of the dentate gyrus.

In Chapter 3, we extended upon these findings by showing that the loss of cholinergic neurons appeared to occur later in development or during adulthood as a

potential failure of these neurons to survive, since 21-day-old IL-2 KO animals did not show signs of a loss of cholinergic neurons of the MS/vDB. Furthermore, the neuronal deficiencies appeared to be selective to the cholinergic neurons of the MS/vDB, as there were no similar decreases of GABAergic neurons in IL-2 KO animals. In the hippocampus, consistent with the reductions in length across the IP-GCL of IL-2 KO compared to wild-type mice, the stereologically estimated cell count of Nissl-stained neuronal somata of IL-2 KO mice was significantly lower than wild-type littermates. Also, the hippocampal neurotrophin levels of BDNF and NGF were significantly decreased and increased, respectively.

Finally, in Chapter 4, the cytokine profile of the IL-2 KO hippocampus was altered with increases in IL-15 and IL-12 and the chemokines MCP-1 and IP-10. These experiments confirmed our hypothesis that loss of IL-2 would result in increased levels of brain IL-15 production. Furthermore, in spite of the known roles of these cytokines and chemokines in T cell trafficking and modulation during brain insult, we were unable to label for elevated levels of the T cells or activated microglia in the hippocampus. The above cytokine profile did not match the serum cytokine levels, suggesting that the changes in cytokines in the hippocampus were likely due to changes in their production in the CNS. Finally, though there was no group alteration in adult GCL neurogenesis, there was a group by gender interaction that appeared to be attributable to the male mice.

Implications

This series of studies is the first to demonstrate that endogenous levels of IL-2 may be an important factor in the late development or survival of neurons in the CNS. This impact on CNS neurons involved not only cell number, but also alterations in levels of trophic factors and brain cytokines. Other studies involving IL-2 and the CNS, to date,

have depended on in vitro models and/or exogenous administration of IL-2 into the CNS in vivo. Whereas studies such as these can yield valuable information, in vitro models often fail to mimic the complexity of an in vivo system (e.g., lack of glia, different organization of neurons, etc.) and the manipulated in vivo models are subject to experimental design complications (e.g., disruption of the BBB, determination of physiologically meaningful doses of exogenously administered cytokines, etc.). We would be remiss, however, to claim that the IL-2 KO model was completely without complications itself (e.g., autoimmunity such as inflammatory bowel disease), but we feel that the most informative way to address a hypothesis is to examine it in multiple different ways. Thus, taken together with the previous studies in the literature, this dissertation reveals an interesting relationship between IL-2 and the septohippocampal system.

The goal of this study was to elicit whether IL-2 KO impacted the septohippocampal system, but not to design a treatment or study a clinical disorder. Thus, at its core, this dissertation was basic research. Whereas the pursuit of knowledge is a noble goal, invariably, a simple question often finds itself in the front of our minds: how is this new knowledge clinically relevant and how can we use it?

Already, IL-2 is being used clinically to treat, or being studied as a treatment for, a number of disorders, including cancer (Atkins et al., 1999; Davis and Gillies, 2003; Fyfe et al., 1995; Guirguis et al., 2002; Parkinson et al., 1990; Rosenberg et al., 1985; Rosenberg et al., 1994), and HIV infection (Armstrong and Kazanjian, 2001; Mitsuyasu, 2001; Smith, 2001). The aforementioned treatments are not, however, completely without side effects. In a landmark study, systemic IL-2 treatment for cancer patients

was shown to induce dose and time-related behavioral and cognitive changes in 22 out of 44 subjects, including spatial and temporal disorientation (Denicoff et al., 1987). Others have also noted cognitive changes in patients undergoing IL-2-treatment (Capuron et al., 2000; Capuron et al., 2001b; Walker et al., 1997).

Of particular relevance to this dissertation, IL-2 immunotherapy can induce cognitive changes, including spatial memory deficits and poor task planning (which may be partially dependent on spatial memory) as early as five days into therapy (Capuron et al., 2001a). Capuron's study utilized neuropsychological batteries to assess these cognitive dysfunctions, but did not investigate the physiological mechanism underlying them. One hypothesis to explain at least part of the cognitive changes observed is that the chronic IL-2 treatment used on these patients may affect the septohippocampal system in a similar way that loss of IL-2 causes changes in IL-2 KO animals. Deletion of IL-2 having a similar physiological effect as administration of exogenous IL-2 may seem counterintuitive; however, in at least one population of neurons (i.e., the septal neurons), IL-2 has potent biphasic modulatory actions on ACh release with low sub-pM IL-2 concentrations enhancing and higher nM concentrations inhibiting release (Hanisch et al., 1993; Seto et al., 1997). Thus, under the proposed hypothesis, a loss of IL-2 could lead to a reduction in the stimulatory action on cholinergic neurons, whereas an overabundance of exogenous IL-2 could inhibit release of ACh. Of course, more investigation would be necessary to clarify that hypothesis.

Caveats and Future Directions

In Study 1, we examined the effects of IL-2 KO on the cholinergic septohippocampal neurons. Many of our findings were extended upon in Study 2. However, we hypothesized that the lack of any change in the cholinergic fiber density in

the hippocampus was likely due to compensatory sprouting, but did not overtly assay for any alterations. Thus, to address this issue further, a study utilizing a stereotaxic injection of an anterograde tracer, such as Phaseolus vulgaris-leucoagglutinin (PHAL), into the MS/vDB area would be necessary. The septohippocampal cholinergic neurons would need to be labeled with a marker for AChE and the length and branching of these double-labeled axons would be characterized. If the septohippocampal cholinergic neurons do undergo compensatory sprouting in IL-2 KO mice, we hypothesize that a detectable increase in branching should occur in those animals vs. wild-type littermates.

Though the majority of cholinergic neurons in the MS/vDB project to the hippocampus (Schwegler et al., 1996b), a small percentage also project to other areas, including the mediodorsal nucleus of the thalamus (Gritti et al., 1998), parietal, cingulate (Gritti et al., 1997), and entorhinal cortices (Alonso and Kohler, 1984). Thus, to accurately claim that the cholinergic neuronal losses observed in the MS/vDB of the IL-2 KO mouse do indeed project to the hippocampus, a study involving a retrograde tracer like Fluoro-Gold injected in the hippocampal projection areas would be necessary. The retrogradely labeled somata of the MS/vDB would need to be double-labeled with ChAT to identify neurons of the cholinergic phenotype and the cell number would be estimated with stereology.

In Study 2, three-week-old IL-2 KO animals did not differ from wild-type littermates in cholinergic cell number. To expand upon this finding, the brains from animals should be examined at one-week intervals from three-week-old mice to adult eight-week-old mice. This will allow us to establish a time course of cholinergic neuronal loss. Furthermore, we would be interested in investigating the nature of the cell

loss. A DNA fragmentation assay, like terminal transferase-mediated dUTP nick-end-labeling (TUNEL), could be used to detect apoptotic cell death and cell death by necrosis could be determined by a simple trypan blue dye exclusion method. Both assays would require immunohistochemical labeling with ChAT to identify the cholinergic somata.

In Study 3, the cytokine and chemokine profile of the IL-2 KO hippocampus was altered compared to wild-type mice. Though we did speculate on the cell types that might produce these cytokines, we did not investigate it further. To identify the potential sources of these cytokines, we could use in situ hybridization to label the mRNA and label the individual cell types with immunohistochemistry. We could expand upon this further by utilizing real time PCR techniques as a semi-quantitative assay of different levels of mRNA expression in homogenates from the hippocampi of IL-2 KO and wild-type mice. These two experiments allow us to identify the cells producing the cytokines and compare the relative levels of mRNA produced for each cytokine between IL-2 KO and wild-type mice.

Several other considerations and questions arise from the data generated from the experiments of this dissertation. First, there is possibility that the reduced levels of ChAT⁺ immunostaining may be due to a loss of the cholinergic phenotype (e.g., inability to produce ACh) rather than cell death. Alterations in the activity of ACh in the hippocampus (e.g., addition of receptor agonists or antagonists) are sufficient to alter learning and memory (for a review see Gold, 2003). Thus, the behavioral deficits observed in the IL-2 KO animals may not be due to cholinergic neuronal loss. Although the staining intensity was uniform (e.g., consistent between and within groups), to test this further, IL-2 KO and wild-type littermate brains should be Nissl-stained to label and

quantify MS/vDB neurons in this brain region. Animals with neuronal loss, should exhibit reduction in total neuronal counts, whereas the neuronal counts would not differ significantly in mice with a loss of phenotype.

Also, considering the difficulty of detection of endogenous IL-2 in the normal CNS, what is the source of the IL-2 in the wild-type brain? Taken together with the unknown non-saturable transport mechanism that allows IL-2 to cross the BBB (Waguespack et al., 1994), and the numerous studies showing cognitive effects of peripherally administered exogenous IL-2 (Capuron et al., 2001a; Denicoff et al., 1987; Lacosta et al., 1999; Walker et al., 1997), an argument can be made that some of the endogenous IL-2 in the CNS could be from the periphery, particularly during development when the BBB is not completely formed. In addition, though we did address the autoimmunity issue of IL-2 KO mice somewhat, we did not completely rule it out as a potential factor in the observed alterations. An experiment that could address both of the above issues would involve crossbreeding the IL-2 KO mice onto an immunodeficient background lacking functional lymphocytes (e.g., RAG-1 knockout mice). Next, an adoptive transfer of normal T lymphocytes from healthy IL-2 wild-type (i.e., non-RAG-1 KO) mice to young (i.e., less than 3-week-old) IL-2 KO/RAG-1 KO or IL-2 wild-type/RAG-1 KO littermate animals would establish a functional immune system, and at the same time restore a major source of peripheral IL-2. Also, another set of IL-2 KO/RAG-1 KO and IL-2 wild-type/RAG-1 KO mice should receive an adoptive transfer of IL-2 KO (i.e., non-RAG-1 KO) lymphocytes to mimic the autoimmune state of normal IL-2 KO mice. Finally, another group of IL-2 KO/RAG-1 KO and IL-2 wild-type/RAG-1 KO mice should receive a sham reconstitution, such that they maintain any

normal levels (or lack thereof in IL-2 KO/RAG-1 KO mice) of endogenous brain IL-2 expression, but remain immunodeficient, thus lacking a peripheral source of IL-2. This experimental design would create a model

- that eliminates the impact of peripheral autoimmunity in the IL-2 wild-type reconstituted IL-2 KO/RAG-1 KO mice;
- in which the aforementioned animals are only lacking a CNS, but not peripheral, source of IL-2;
- in which the IL-2 wild-type reconstituted IL-2 wild-type/RAG-1 KO mice would have a functional peripheral immune system and normal endogenous IL-2 expression in the brain;
- in which the IL-2 KO reconstituted IL-2 KO/RAG-1 KO mice are similar in phenotype to the normal IL-2 KO mouse (i.e., peripheral autoimmunity);
- in which the IL-2 KO reconstituted IL-2 wild-type/RAG-1 KO mice would have normal brain IL-2 expression, but autoimmunity caused by the peripheral IL-2 KO T lymphocytes; and
- in which the sham reconstituted IL-2 KO/RAG-1 KO mice lack any source of IL-2 and the sham reconstituted wild-type/RAG-1 KO mice lack a peripheral source of IL-2, with neither of the two models succumbing to autoimmunity.

Thus, if endogenous brain IL-2 is important in septohippocampal development and/or maintenance from the age of three-weeks to adulthood, then we should observe deficits in the septohippocampal system similar to those noted in this dissertation in all reconstitution and sham models above on an IL-2 KO/RAG-1 KO background. If the peripheral source of IL-2 is more important for septohippocampal physiology, then the structural and physiological alterations in this dissertation should be rescued in the above IL-2 wild-type reconstituted cases, but not IL-2 KO or sham reconstituted animals. If autoimmunity is the determining factor, then IL-2 KO reconstituted, but not sham reconstituted, animals will all exhibit deficits in the septohippocampal system regardless of background. This proposed experiment combining IL-2 KO strains, immunodeficient

strains, and reconstitution allows us to control for several immunological and genetic factors without disruption of the BBB.

Concluding Remarks

In the peripheral immune system, the complex interplay and interactions of the various cytokines often have redundant, supportive, or even oppositional roles. This complexity allows for a system of compensatory and regulatory control of the immune response. Similar overlaps and checks and balances are also present in the CNS, so understanding how these intricate systems interact can prove daunting. Nevertheless, elucidating the relationship between the brain and immune system molecules may have profound clinical utility. The IL-2 KO mouse is a complicated model to study a cytokine brain interaction and, in this dissertation, we have attempted to simplify the model by not experimentally manipulating it. Our goal was to lay groundwork for future studies on this topic, whereby we hope to more fully understand the precise mechanisms by which IL-2 alters brain physiology.

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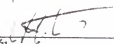
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BIOGRAPHICAL SKETCH

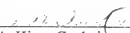
Ray Dennis Beck, Jr. was born in York, PA, to Daisy and the appropriately named Ray Dennis Beck, Sr. Realizing the error of his ways early in life, Ray moved to Houston, TX, at the age of two, lending credence to the old Texas saying, “I wasn’t born in Texas, but I got here as fast as I could.” He attended Oak Ridge High School until the age of 16. Ranked 14 overall in his class, he elected to forego his senior year to attend Simon’s Rock College of Bard in Great Barrington, MA. Whereas some 16-year-old adolescents are mature enough to pursue a college education – while resisting distractions like geographically convenient buildings filled with members of the opposite sex, a ready supply of fermented beverages, and complete lack of parental supervision – Ray was not. He returned to Houston after his first year of college with a less than stellar GPA intent on taking a year off from school. During this “year” (comprised of ~1,825 days), he held various jobs ranging from perfume salesman to waiter at various restaurants. During one of the latter jobs, he met his present wife, Laura Frakey. Inspired by her enthusiasm for education, Ray enrolled in the University of Houston, while maintaining full-time employment to pay for school. He graduated *cum laude* with a B.S. in biology. Ray and Laura moved to Gainesville, FL, to attend University of Florida graduate programs in neuroscience and psychology, respectively. With the completion of his Ph.D., Ray is proud to be one of the most educated high school dropouts that anyone is ever likely to meet. Ray is an avid follower of movies and his other interests can be classified as “all things geeky” (e.g., computers, roleplaying, video games, etc.).

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



John M. Petitto, Chair
Professor of Neuroscience

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Michael A. King, Cochair
Associate Scientist of Neuroscience

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Professor of Neuroscience

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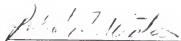
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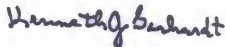
Mark A. Atkinson
Professor of Pathology, Immunology and
Laboratory Medicine

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 2004



Dean, College of Medicine



Dean, Graduate School